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Ethanol Experience Induces Metaplasticity of NMDA Receptor-Mediated Transmission in Ventral Tegmental Area Dopamine Neurons

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by

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Dedication

For my mother

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Ethanol Experience Induces Metaplasticity of NMDA Receptor-Mediated Transmission in Ventral Tegmental Area Dopamine Neurons

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Addiction is thought to arise, in part, from a maladaptive learning process in which enduring memories of drug-related experiences are formed, resulting in persistent and uncontrollable drug-seeking behavior. However, it is well known that both acute and chronic alcohol (ethanol) exposures impair various types of learning and memory in both humans and animals. Consistent with these observations, both acute and chronic exposures to ethanol suppress synaptic plasticity, the major neural substrate for learning and memory, in multiple brain areas. Therefore, it remains unclear how powerful memories associated with alcohol experience are formed during the development of alcoholism.

The mesolimbic dopaminergic system is critically involved in the learning of information related to rewards, including drugs of abuse. Both natural and drug rewards, such as ethanol, cause release of dopamine in the nucleus accumbens and other limbic structures, which is thought to drive learning by enhancing synaptic plasticity. Accumulating evidence indicates that plasticity of glutamatergic transmission onto

dopamine neurons may play an important role in the development of addiction. Plasticity of NMDA receptor (NMDAR)-mediated transmission may be of particular interest, as NMDAR activation is necessary for dopamine neuron burst firing and phasic dopamine release in projection areas that occurs in response to rewards or reward-predicting stimuli. NMDAR plasticity may, therefore, drive the learning of stimuli associated with rewards, including drugs of abuse.

This dissertation finds that repeated *in vivo* ethanol exposure induces a metaplasticity of NMDAR-mediated transmission in mesolimbic dopamine neurons, expressed as an increased susceptibility to the induction of NMDAR LTP. Enhancement of NMDAR plasticity results from an increase in the potency of inositol 1,4,5-trisphosphate (IP_3) in producing the facilitation of action potential-evoked Ca^{2+} signals critical for LTP induction. Interestingly, amphetamine exposure produces a similar enhancement of IP_3R function, suggesting this neuroadaptation may be a common response to exposure to multiple drugs of abuse. Additionally, ethanol-treated mice display enhanced learning of cues associated with cocaine exposure. These findings suggest that metaplasticity of NMDAR LTP may contribute to the formation of powerful memories related to drug experiences and provide an important insight into the learning component of addiction.

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Chapter 1: Introduction

1.1 Reward learning

For all animals, the ability to successfully obtain resources that ensure health and reproduction is critically important for both individual and species survival. These biologically relevant resources are known as "primary rewards" and are defined by their tendency to evoke approach and consummatory behavior. In addition, primary rewards produce feelings of pleasure and function to initiate and maintain behavior during learning (W. Schultz, 1998). In this sense, primary rewards function behaviorally as reinforcers, defined as any stimulus that increases the probability of a behavior that results in delivery of that stimulus (B. F. Skinner, 1953).

Over the course of evolution selective pressures have resulted in the development of specific reward-learning mechanisms which allow organisms to continually adapt their behavioral responses to a changing environment as they attempt to secure essential resources, such as food and potential mates (A. Dickinson, 1980; K. L. Hollis, 1997). Pioneering work by Ivan Pavlov described a form of reward learning now known as "classical" or "Pavlovian" conditioning, in which animals form unconscious associations between neutral stimuli or cues that are repeatedly paired with primary rewards (I. P. Pavlov, 1960). Through such associative processes animals may learn about causal relationships in their environment and thus acquire predictive information conferring survival advantages when competing over scarce resources in a natural environment. For

example, extensive studies have shown that Pavlovian conditioning contributes to food recognition early in development, confers advantages in territorial defense and defense against dominant conspecifics and predators, and increases reproductive success and efficiency (K. L. Hollis, 1997).

1.1.1 The dopaminergic system and brain stimulation reward

The search for the neural systems responsible for processing information related to rewards began in the 1950's when Olds and Milner reported that intracranial electrical stimulation to areas of the lateral hypothalamus (LH) is highly rewarding in rats (J. Olds and P. Milner, 1954; J. Olds, 1958). In the original study, Olds and Milner found that if they gave stimulation only when the animal was in one corner of the cage the animal would continually return to that location, as if "coming back for more". (J. Olds and P. Milner, 1954). This behavioral response would later be termed conditioned place preference (CPP), and this observation led Olds to speculate that LH stimulation activated brain circuits mediating the rewarding properties of natural rewards, such as food and sex. This theory was supported by the finding that intracranial electrical stimulation can function similarly to natural rewards, as a positive reinforcer of a variety of behaviors including lever-pressing and maze-running (J. Olds, 1958). In addition, the rewarding properties of brain stimulation were found to be extremely powerful. Animals will ignore food in favor of lever-pressing for intracranial stimulation to the point of starvation and willingly subject themselves to painful foot-shocks in order to gain access to the self-stimulation lever (J. Olds, 1958; R. A. Wise, 1996a). Subsequent mapping of

electrical self-stimulation loci identified a fiber tract known as the median forebrain bundle (MFB) as the important site within the LH for self-stimulation, and the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), midbrain dopaminergic structures, as additional sites capable of supporting high levels of self-stimulation (A. Routtenberg and C. Malsbury, 1969; T. J. Crow, 1972; D. Corbett and R. A. Wise, 1980; R. A. Wise, 2005). Additional work focusing on the dopaminergic system found that either lesions of midbrain dopamine (DA) structures or pharmacological blockade of DA transmission significantly reduces the rewarding properties of self-stimulation (A. S. Lippa et al., 1973; G. Fouriezos and R. A. Wise, 1976; K. B. Franklin and S. N. McCoy, 1979; P. Zarevics and P. E. Setler, 1979; H. C. Fibiger et al., 1987). These findings combined with further anatomical studies led to the conclusion that MFB stimulation is rewarding due to its ability to activate the midbrain DA system (D. Corbett and R. A. Wise, 1980; R. A. Wise, 1996b) establishing a central role for DA systems in the processing of reward. Subsequent studies have supported this conclusion by demonstrating a role for DA systems in mediating reward associated with food (R. A. Wise et al., 1978; F. Jenck et al., 1987), water (G. J. Gerber et al., 1981) and sex (J. G. Pfaus and A. G. Phillips, 1989; D. F. Fiorino et al., 1997).

1.1.2 The dopaminergic system and addictive drugs

Addictive drugs are characterized by their highly rewarding properties. Like natural rewards, drugs induce subjective feelings of pleasure and learning of cues and contexts that predict their availability (S. E. Hyman et al., 2006). Consequently, it has

been hypothesized that addictive substances exert their powerful motivational effects by usurping brain systems evolved for learning of information related to natural rewards. Early animal studies confirmed that, similarly to natural rewards and intracranial brain stimulation, addictive drugs are behaviorally reinforcing. Animals given access to intravenous drug self-administration quickly increase their intake with experience (R. A. Wise, 1996a), and numerous addictive drugs can elicit a robust CPP for the environments in which the drug is experienced (T. M. Tzschentke, 1998). As with humans, animals will prefer drugs over natural rewards, and will continue drug-seeking behavior regardless of interruptions or obstacles (S. E. Hyman et al., 2006). It is therefore somewhat unsurprising that much of the research on the neural substrates of drug reward and addiction have focused on the same dopaminergic structures implicated in other reward-related behaviors.

In particular, the mesocorticolimbic DA pathway, consisting of dopaminergic neurons in the ventral tegmental area (VTA) and their projections to target structures including the nucleus accumbens (NAc) and prefrontal cortex (PFC), is thought to be a key component of the circuitry mediating drug reward and addiction (R. A. Wise, 1996a; M. Melis et al., 2005). Indeed, the rewarding properties of all addictive substances are believed to derive from their shared ability to increase DA levels in the NAc (G. Di Chiara and A. Imperato, 1988; M. Melis et al., 2005). Consistent with this idea, lesions of the NAc or treatment with DA receptor antagonists can block the rewarding effects and self-administration of drugs of abuse in animals (R. A. Wise and P. P. Rompre, 1989). Furthermore, animals will readily self-administer many drugs, including ethanol,

cocaine, amphetamine, opiates, and cannabis, directly into either the VTA or NAc, highlighting the central role of these structures in drug reward (S. Ikemoto and R. A. Wise, 2004).

1.2 THE MESOCORTICOLIMBIC DOPAMINE SYSTEM

The mesocorticolimbic dopamine system consists of dopamine neurons within the VTA and their projections to limbic and cortical areas, including the NAc, amygdala, and prefrontal, cingulate and entorhinal cortices (M. Melis et al., 2005). Aside from DA neurons, which account for approximately 55% of the population, the VTA also contains a significant proportion of γ-aminobutyric acid (GABA)ergic neurons (D. B. Carr and S. R. Sesack, 2000; E. B. Margolis et al., 2006), as well as a third population of unidentified neurons, which are neither dopaminergic or GABAergic though their electrophysiological properties are similar to those of DA neurons (D. L. Cameron et al., 1997; M. A. Ungless et al., 2004). There is some evidence to suggest these are glutamatergic projection neurons, as VTA stimulation can produce glutamatergic excitatory postsynaptic potentials (EPSPs) in target structures (A. Lavin et al., 2005). However, recent evidence also suggests that some VTA DA neurons co-release glutamate, leaving the classification of these nondopaminergic neurons unresolved (N. Chuhma et al., 2004; G. D. Stuber et al., 2010).

1.2.1 Dopamine neuron firing patterns

In vivo, DA neurons display two general firing patterns, shifting between slow, irregular, single spike firing (tonic activity) and short bursts of action potentials (phasic activity) (A. A. Grace and B. S. Bunney, 1984b, a; P. G. Overton and D. Clark, 1997). These differential modes of activity in turn produce distinct patterns of DA release in target structures and are thought to subserve different neural functions (W. Schultz, 2007). Tonic activity maintains a low level of extrasynaptic DA in downstream areas that changes slowly, on a timescale of minutes, and is theorized to play an enabling role in motor, cognitive and motivational systems (W. Schultz, 2007). Phasic activity produces large and fast changes in downstream DA concentrations, on a timescale of milliseconds, and is thought to function as a value signal in reward learning and a salience signal contributing to motivational control (K. C. Berridge and T. E. Robinson, 1998; W. Schultz, 1998; E. S. Bromberg-Martin et al., 2010).

DA neuron bursts exhibit increasing interspike intervals and decreasing spike amplitudes and are frequently terminated by periods of silence (A. A. Grace and B. S. Bunney, 1984b). These bursts are triggered by excitatory synaptic inputs, as they can be evoked *in vivo* by electrical stimulation of glutamatergic inputs or microiontophoretic application of glutamate (P. G. Overton and D. Clark, 1997; S. B. Floresco et al., 2003). N-methyl d-aspartate (NMDA) receptors appear to play a predominant role in mediating the transition to burst firing as NMDA but not α-amino-3hydroxyl-5-methyl-4isoxazole-propionate (AMPA) receptor antagonists block burst firing (P. Overton and D. Clark, 1992; K. Chergui et al., 1994b; Z. Y. Tong et al., 1996a). Additionally, a recent study

has shown that genetic knockout of NMDARs in DA neurons greatly reduces burst firing (L. S. Zweifel et al., 2009).

In vitro, DA neurons spontaneously fire single spikes in a regular, pacemaking manner from 0.5-5 Hz (A. A. Grace and B. S. Bunney, 1984a; S. W. Johnson and R. A. North, 1992a). As afferent connections to the VTA are severed in the brain slice preparation, this pacemaking activity pattern is believed to reflect intrinsic properties of DA neurons in the absence of synaptic inputs (S. T. Kitai et al., 1999; M. Melis et al., 2005). Work in acute brain slices attempting to identify the main ionic conductances responsible for pacemaking activity in DA neurons has yielded complex results, as many earlier studies incorrectly assumed SNc and VTA DA neurons to be a homogeneous population. More recent work has clearly shown that this is not the case. On the contrary, there are important differences in the contributions of various conductances to DA neuron pacemaking between the SNc and VTA, and even within the VTA (J. Wolfart et al., 2001; H. Neuhoff et al., 2002; C. S. Chan et al., 2007).

The hyperpolarization-activated cation channel (I_h) is a mixed Na^+/K^+ channel implicated in spontaneous pacemaking in DA neurons and multiple other types of neurons throughout the CNS (A. A. Grace and S. P. Onn, 1989; S. W. Johnson and R. A. North, 1992a; H. C. Pape, 1996; H. Neuhoff et al., 2002). As they are activated by hyperpolarization, I_h channels in DA neurons are activated following AP firing by the afterhyperpolarization (AHP) and contribute to pacemaking by depolarizing the neuron towards AP threshold. In line with this, I_h blockers have been shown to slow pacemaking frequency in both SNc and VTA DA neurons in some studies (T. Okamoto et al., 2006; J.

McDaid et al., 2008; M. J. Beckstead and T. J. Phillips, 2009), however others have suggested that I_h is not a major contributor to pacemaking in the VTA, as it is in the SNc (H. Neuhoff et al., 2002; Z. M. Khaliq and B. P. Bean, 2010).

The role of L-type Ca^{2+} channels in DA neuron pacemaking has also been extensively studied. L-type Ca^{2+} channel activity is thought to maintain a subthreshold membrane potential oscillation that underlies spontaneous firing (P. Durante et al., 2004; C. S. Chan et al., 2007). Indeed, specific L-type Ca^{2+} channel antagonists block pacemaking activity in SNc DA neurons (N. B. Mercuri et al., 1994; C. S. Chan et al., 2007). However, spontaneous firing in VTA DA neurons is unaffected by L-type Ca^{2+} channel antagonists (C. S. Chan et al., 2007) or replacing Ca^{2+} in the extracellular solution (Z. M. Khaliq and B. P. Bean, 2010), demonstrating that L-type Ca^{2+} channels do not play a predominant role in pacemaking in the VTA. In these recent studies in which neither I_h blockade or Ca^{2+} replacement affected spontaneous firing rates in VTA DA neurons, the authors instead found evidence that pacemaking depends upon two distinct Na^{+} currents; a voltage-independent leak current active during the interspike interval and a separate voltage-dependent current activated at slightly more depolarized voltages that drives the neuron toward the AP threshold (C. S. Chan et al., 2007; Z. M. Khaliq and B. P. Bean, 2010).

1.2.2 Afferent modulation of DA neuron firing

Afferent inputs exert a powerful regulatory influence on the firing patterns of VTA DA neurons (A. A. Grace and B. S. Bunney, 1984b; A. A. Grace et al., 2007). The VTA is innervated by glutamatergic, GABAergic, cholinergic, noradrenergic, serotonergic and peptidergic fibers originating in numerous structures extending in a somewhat continuous formation from the prefrontal cortex to the brainstem (Figure 1) (S. Geisler and D. S. Zahm, 2005; M. Melis et al., 2005). Information related to rewards and reward-predicting cues is carried by all sensory modalities and is heavily influenced by internal states and other psychological factors. The complexity and diversity of inputs to DA neurons suggests that, at least in part, the integration of this wide variety of information takes place at the level of the VTA itself (S. Geisler et al., 2007).

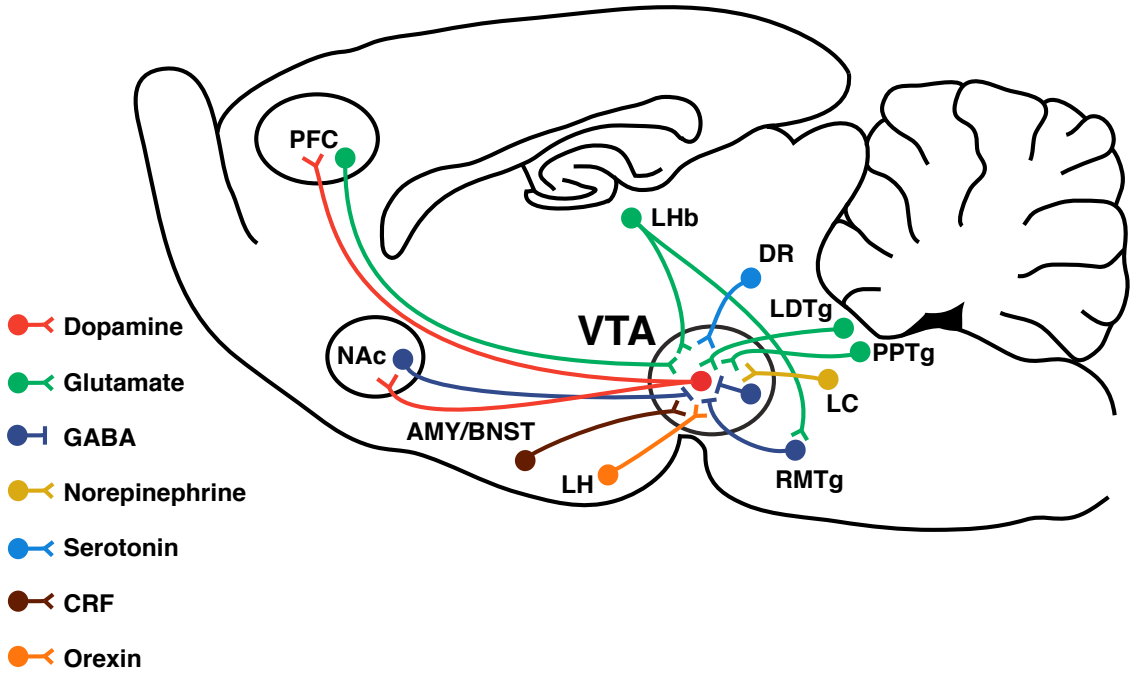


Figure 1. Mesocorticolimbic dopamine system circuitry.

Simplified schematic diagram highlighting the major inputs and outputs of the VTA. Noradrenergic, serotonergic, and peptidergic projections shown here also contain glutamatergic projections. NAc, nucleus accumbens; PFC, prefrontal cortex; LHb, lateral habenula; DR, dorsal raphe nucleus; LDTg, laterodorsal tegmental nucleus; PPTg, pedunculopontine tegmental nucleus; LC, locus coeruleus; RMTg, rostromedial tegmental nucleus; LH, lateral hypothalamus; AMY, amygdala; BNST, bed nucleus of the stria terminalis.

1.2.2.1 Glutamatergic afferents

Burst firing in DA neurons is thought to be primarily mediated by activation of glutamatergic inputs (P. G. Overton and D. Clark, 1997). In addition, glutamatergic transmission in the VTA has been implicated in many of the actions of drugs of abuse (J. A. Kauer and R. C. Malenka, 2007; S. Geisler and R. A. Wise, 2008). As a result, the sources and functional roles of these glutamatergic afferents have been extensively studied. The most significant of these include the medial prefrontal cortex (PFC), lateral hypothalamus, lateral habenula (LHb), pedunculopontine tegmental nucleus (PPTg) and laterodorsal tegmental nucleus (LDTg), however there are numerous other glutamatergic afferents to the VTA originating in almost every area projecting to the VTA (S. Geisler et al., 2007)

The medial PFC contains the largest number of glutamatergic neurons projecting to the VTA of any brain region (S. Geisler and R. A. Wise, 2008). The majority of these neurons are found within the prelimbic and dorsal peduncular cortices, with a smaller number of VTA projecting neurons found in the infralimbic, cingulate, orbital and insular cortices (S. Geisler et al., 2007). Despite the large number of direct connections between PFC and DA neurons, the role of the PFC in regulating DA neuron firing behavior is somewhat complex (S. Geisler and R. A. Wise, 2008). While stimulation of the PFC does induce burst firing in DA neurons, many DA neurons also exhibit inhibitions in response to PFC stimulation (R. F. Gariano and P. M. Groves, 1988; Z. Y. Tong et al., 1996b). In addition, DA neuron bursts evoked by electrical stimulation of the PFC frequently show latencies too long to be monosynaptic, suggesting that PFC regulation of DA neuron activity occurs through a polysynaptic mechanism involving other brain

regions (S. Geisler and R. A. Wise, 2008). Furthermore, an *in vivo* study in anesthetized rats found that PFC and DA neuron activity covaries, though positively in some neurons and negatively in others (M. Gao et al., 2007), suggesting that the PFC modulates DA neuron firing through both excitatory and inhibitory pathways.

Though they represent a relatively small proportion of glutamatergic afferents to the VTA, the PPTg and LDTg nuclei are believed to play a prominent role in DA neuron burst firing (S. Geisler and R. A. Wise, 2008). *In vivo* recordings in rats have shown that stimulation of the PPTg increases burst firing in VTA DA neurons and DA release in target structures (S. B. Floresco et al., 2003), while chemical inactivation of the PPTg reduces DA neuron responses to reward-conditioned cues (W. X. Pan and B. I. Hyland, 2005). Similarly, activation of the LDTg is critical for burst firing in VTA DA neurons, as LDTg inactivation almost entirely blocks burst firing in anesthetized rats and converts DA neuron firing to regular, pacemaking activity closely resembling that seen in the *in vitro* slice preparation (D. J. Lodge and A. A. Grace, 2006). Interestingly, this study also found that following LDTg inactivation, neither stimulation of the PPTg or iontophoretic application of glutamate were able to evoke burst firing in DA neurons, suggesting that tonic LDTg activity interacts with other inputs by enabling the induction of burst firing (D. J. Lodge and A. A. Grace, 2006). However, it is important to note that the LDTg also sends a prominent cholinergic input to VTA DA neurons, and this study was not able to determine which neurotransmitter is responsible for the gating of burst firing by the LDTg.

The glutamatergic projection from the LHb to the VTA has recently been shown to play an interesting role in regulating DA neuron firing. *In vivo* recordings in primates have shown that lateral habenula neurons increase firing in response to unrewarded behavioral trials and decrease firing in response to rewarded trials (M. Matsumoto and O.

Hikosaka, 2007). Thus, LHb activation is thought to encode negative reward value (M. Matsumoto and O. Hikosaka, 2007). These inputs are thought to inhibit DA neuron firing through activation of GABAergic interneurons in the VTA, as stimulation of LHb decreases population activity of VTA DA neurons in a $GABA_A$ receptor dependent manner (G. R. Christoph et al., 1986; H. Ji and P. D. Shepard, 2007). Consistent with this idea, there is evidence that LHb projections to the VTA synapse primarily on GABAergic neurons (S. Geisler and R. A. Wise, 2008; K. Brinschwitz et al., 2010). However, a recent study suggests that inhibition of DA neurons by LHb activation is also mediated by the projection from LHb to the newly identified GABAergic rostromedial tegmental nucleus (RMTg), which in turn projects heavily to the VTA (Figure 1) (T. C. Jhou et al., 2009).

Finally, numerous other areas send glutamatergic projections to the VTA, though the functional impact of these inputs on DA neuron firing patterns is not well known. Most notably, the lateral hypothalamus contains a large number of glutamatergic projection neurons targeting the VTA (S. Geisler et al., 2007). Smaller glutamatergic projections to the VTA include the lateral preoptic area, superior colliculus, mesopontine central gray, ventral pallidum, mesopontine reticular formation, medial preoptic area, median raphe, and dorsal raphe (S. Geisler et al., 2007).

1.2.2.2 GABAergic afferents

GABAergic neurons intrinsic to the VTA provide a major source of inhibitory input onto DA neurons (S. W. Johnson and R. A. North, 1992a; S. C. Steffensen et al., 1998). These GABAergic neurons send collaterals throughout the VTA and their spontaneous

activity maintains an inhibitory tone on DA neurons that can be detected in the slice preparation (S. W. Johnson and R. A. North, 1992a; N. Omelchenko and S. R. Sesack, 2009). In addition, DA neurons receive significant "long-loop" GABAergic inhibitory feedback from the ventral pallidum (VP) and the medium spiny neurons of the NAc (L. C. Conrad and D. W. Pfaff, 1976; R. Spanagel and F. Weiss, 1999; S. Geisler and D. S. Zahm, 2005). Finally, recent work has identified the RMTg as the source of another important GABAergic input onto VTA DA neurons (T. C. Jhou et al., 2009). Neurons in this structure, located just caudal to the VTA, are activated by aversive stimuli, inhibited by rewards and are thought to mediate the inhibition of DA neurons by the LHb (T. C. Jhou et al., 2009).

GABAergic inputs hyperpolarize DA neurons through activation of $GABA_A$ or $GABA_B$ receptors, though evidence suggests that inputs from local VTA neurons selectively activate $GABA_A$ receptors, while extrinsic inputs activate $GABA_B$ receptors (S. W. Johnson and R. A. North, 1992a; S. Sugita et al., 1992). *In vitro* and *in vivo* studies have shown that activation of GABA receptors can decrease both the average firing rate and burst firing of VTA DA neurons (H. R. Olpe et al., 1977; V. Seutin et al., 1994; S. Erhardt et al., 2002).

1.2.2.3 Cholinergic afferents

The major cholinergic input to VTA DA neurons originates in the PPTg and the LDTg, two mesopontine nuclei also known to send important glutamatergic projections to the VTA (U. Maskos, 2008). DA neurons express both ionotropic nicotinic and

metabotropic muscarinic acetylcholine receptors (P. B. Clarke and A. Pert, 1985) and double-labelling tract-tracing studies have identified excitatory cholinergic synapses from the LDTg and PPTg on VTA DA neurons (K. Semba and H. C. Fibiger, 1992; M. Garzon et al., 1999). Consistent with the anatomical data, application of acetylcholine depolarizes VTA DA neurons in brain slice experiments (P. Calabresi et al., 1989), while *in vivo*, nicotine administration increases burst firing in VTA DA neurons (J. Grenhoff et al., 1986). Interestingly, this study also found that nicotinic antagonists inhibit firing in DA neurons, suggesting the presence of a tonic acetylcholine input. More recent *in vivo* work has shown that burst firing is significantly reduced in VTA DA neurons in mice lacking the nicotinic β2 receptor subunit, while this deficit can be rescued by re-expressing the β2 subunit selectively in DA neurons (M. Mameli-Engvall et al., 2006). These findings, in conjunction with the previously described study that found a lack of burst firing following LDTg inactivation (D. J. Lodge and A. A. Grace, 2006), have been interpreted as evidence that cholinergic input to the VTA enables the initiation of burst firing by other, presumably glutamatergic, inputs (U. Maskos, 2008). However, as mentioned above, the study involving LDTg inactivation was not able to determine whether it was cholinergic or glutamatergic inputs from the LDTg that are necessary to enable burst firing.

1.2.2.4 Noradrenergic afferents

The VTA receives noradrenergic inputs from the locus coeruleus (LC) and the A5 area of the medulla, a brainstem region associated with homeostatic functions (S. Geisler

and D. S. Zahm, 2005; C. A. Mejias-Aponte et al., 2009). Ultrastructural studies have shown these inputs synapse onto DA neurons (L. A. Liprando et al., 2004), which express both α-1 and α-2 adrenergic receptors (L. S. Jones et al., 1985; A. Lee et al., 1998). *In vivo* blockade of these receptors reduces burst firing and regularizes tonic firing (J. Grenhoff and T. H. Svensson, 1993), while electrical stimulation of the LC results in an excitation of DA neurons followed by an inhibition (J. Grenhoff et al., 1993). Data from *in vitro* studies suggests that these effects are due to multiple noradrenergic effects including an α-1 receptor dependent depolarization (J. Grenhoff et al., 1995), an inhibition of metabotropic glutamate receptor (mGluR)-mediated inhibitory postsynaptic potentials (IPSPs) (C. A. Paladini et al., 2001), and an α-1 receptor dependent hyperpolarization (C. A. Paladini and J. T. Williams, 2004). This hyperpolarization is mediated by intracellular Ca^{2+} release and subsequent activation of the small conductance Ca^{2+}-activated potassium channel (SK) (C. A. Paladini and J. T. Williams, 2004).

Functionally, noradrenergic inputs to the VTA are thought to encode information related to the arousal state of the animal, in the case of the LC, or the physiological state, in the case of the A5 area (C. A. Mejias-Aponte et al., 2009). In addition, these projections may be involved in the stress response as both LC and DA neurons are activated by various stressful stimuli (C. A. Mejias-Aponte et al., 2009).

1.2.2.5 Serotonergic afferents

The dorsal raphe (DR) nucleus sends a substantial serotonergic projection to the VTA (R. P. Vertes, 1991). These afferents are known to form synapses on both DA and

non-DA neurons within the VTA (D. Herve et al., 1987) and are thought to act through multiple different receptor subtypes to exert complex effects on DA neurons (F. J. White, 1996). *In vivo* studies using electrical stimulation of the DR have found a predominantly inhibitory effect on DA neuron activity (M. D. Kelland et al., 1993; J. Gervais and C. Rouillard, 2000). However, *in vitro* slice experiments have found serotonin to have both excitatory and inhibitory actions on DA neurons (F. J. White, 1996; M. Melis et al., 2005). Further adding to this complexity, serotonin can also indirectly modulate DA neuron activity through presynaptic effects on GABAergic (D. L. Cameron and J. T. Williams, 1994) and glutamatergic transmission in the VTA (S. Jones and J. A. Kauer, 1999).

1.2.2.5.6 Peptidergic afferents

The VTA contains terminals releasing the opioid peptides β-endorphin, enkephalin and dynorphin (V. M. Pickel et al., 1993; M. Garzon and V. M. Pickel, 2002). The β-endorphin-containing projections arise in the arcuate nucleus of the hypothalamus, while the dynorphin releasing terminals in the VTA originate in the NAc, amygdala and lateral hypothalamus (J. H. Fallon et al., 1985; H. Khachaturian et al., 1985; L. M. Oswald and G. S. Wand, 2004). Both endorphins and enkephalins activate μ-opioid receptors. Along with other μ-opioid receptors agonists, including opiate drugs such as morphine, these endogenous opioids increase DA neuron firing and subsequent DA release in the NAc (K. Gysling and R. Y. Wang, 1983; L. G. Latimer et al., 1987; G. Di Chiara and A. Imperato, 1988; P. Leone et al., 1991; M. Yoshida et al., 1993). This

excitatory effect of μ-opioid receptor agonists on DA neurons is thought to be an indirect disinhibition resulting from inhibition of VTA GABA neurons (K. Gysling and R. Y. Wang, 1983; S. W. Johnson and R. A. North, 1992b). Data from ultrastructural studies are consistent with this theory, as μ-opioid receptors are predominantly expressed on non-dopaminergic cell bodies and terminals in the VTA (M. Garzon and V. M. Pickel, 2001). Unsurprisingly, injection of μ-opioid receptor agonists directly into the VTA is reinforcing and produces conditioned place preference (A. G. Phillips and F. G. LePiane, 1980).

Dynorphin is a selective κ-opioid receptor agonist that acts in opposition to μ-opioid receptor agonists on DA neuron activity and DA release (C. Chavkin et al., 1982; R. Spanagel et al., 1992; J. T. Williams et al., 2001). In contrast to the μ-opioid receptor agonists, dynorphin or κ-opioid receptor agonists inhibit DA release by activating receptors on both DA axon terminals and cell bodies (R. Spanagel et al., 1990; E. B. Margolis et al., 2003; C. P. Ford et al., 2007). In addition, microinjection of κ-opioid receptor agonists into the VTA produces a conditioned place aversion (R. Bals-Kubik et al., 1993)

VTA inputs arising from the LH release the arousal and feeding-related neuropeptides orexin A and orexin B from both passing fibers and synaptic contacts on DA and GABA neurons (J. J. Balcita-Pedicino and S. R. Sesack, 2007). Orexins act through two different G protein-coupled receptors expressed in the VTA, orexin receptor type 1 and type 2, to increase the firing rate of both DA and GABA neurons (T. M. Korotkova et al., 2003). However, the net effect of orexin release in the VTA is an

increase in DA release in target structures (M. Narita et al., 2006; N. M. Vittoz and C. W. Berridge, 2006). *In vitro* experiments have demonstrated that brief application of orexin causes a transient increase in NMDA receptor-mediated currents in DA neurons (S. L. Borgland et al., 2006). As NMDA receptor activation drives burst firing in DA neurons, this potentiation may contribute to the effects of orexins on DA release (A. Bonci and S. Borgland, 2009). Interestingly, numerous studies have found a role for orexins in reward and drug-related behaviors including self-administration, reinstatement of CPP or drug-seeking and behavioral sensitization (A. Bonci and S. Borgland, 2009).

Another important peptidergic input to the VTA comes from neurons containing the stress-related peptide corticotropin releasing factor (CRF). These inputs arise from the bed nucleus of the stria terminalis (BNST), the paraventricular nucleus of the hypothalamus (PVN) and the central nucleus of the amygdala (D. Rodaros et al., 2007) and synapse directly onto DA neurons (P. Tagliaferro and M. Morales, 2008). Numerous *in vitro* studies have shown that CRF exerts complex effects on DA neurons through two different receptors ($CRFR_1$ and $CRFR_2$), coupled to multiple G proteins and intracellular signaling pathways. For example, activation of CRFR1 increases DA neuron firing through a PKC-dependent mechanism (M. J. Wanat et al., 2008), while activation of $CRFR_2$ can potentiate NMDA receptor-mediated currents and enhance Ca^{2+} signaling through PKC- and PKA-dependent mechanisms, respectively (M. A. Ungless et al., 2003; A. C. Riegel and J. T. Williams, 2008). Consistent with these findings, stress causes release of both CRF in the VTA (R. A. Wise and M. Morales, 2010) and DA in NAc and prefrontal cortex (A. M. Thierry et al., 1976; F. M. Inglis and B. Moghaddam, 1999). It

is, therefore, unsurprising that CRF and its interactions with the DA system have been heavily implicated in addiction and drug-seeking behaviors (M. Heilig and G. F. Koob, 2007; R. A. Wise and M. Morales, 2010).

1.2.3 DA neuron autoregulation

In addition to the numerous afferent inputs, autoregulation mediated by local somatodendritic DA release plays an important role in modulating DA neuron firing activity (P. M. Groves et al., 1975; A. Adell and F. Artigas, 2004). Both *in vitro* and *in vivo* studies have shown that DA is released within the VTA where it inhibits DA neuron firing through actions on the D_2-type DA receptors (P. M. Beart and D. McDonald, 1982; F. J. White and R. Y. Wang, 1984; G. L. Bernardini et al., 1991). Stimulation of D_2 receptors hyperpolarizes DA neurons and reduces firing by activating the G protein-gated inwardly rectifying K^+ (GIRK) channel (M. G. Lacey et al., 1987). This D_2 receptor-mediated hyperpolarization appears to maintain a tonic inhibition on DA neuron activity, as microdialysis studies have shown that D_2 receptor antagonists increase DA neuron firing rates *in vivo* (F. J. White and R. Y. Wang, 1984; N. N. Chen and W. H. Pan, 2000). Additionally, stimulation of the VTA *in vitro* results in a D_2-dependent inhibitory post-synaptic potential (IPSP) in DA neurons, suggesting that DA autoinhibition can occur directly through synaptic transmission (M. J. Beckstead et al., 2004). Indeed, ultrastructural studies have shown that both axodendritic and dendrodendritic synapses between DA neurons are found in the VTA (V. E. Bayer and V. M. Pickel, 1990).

1.2.4 VTA projections

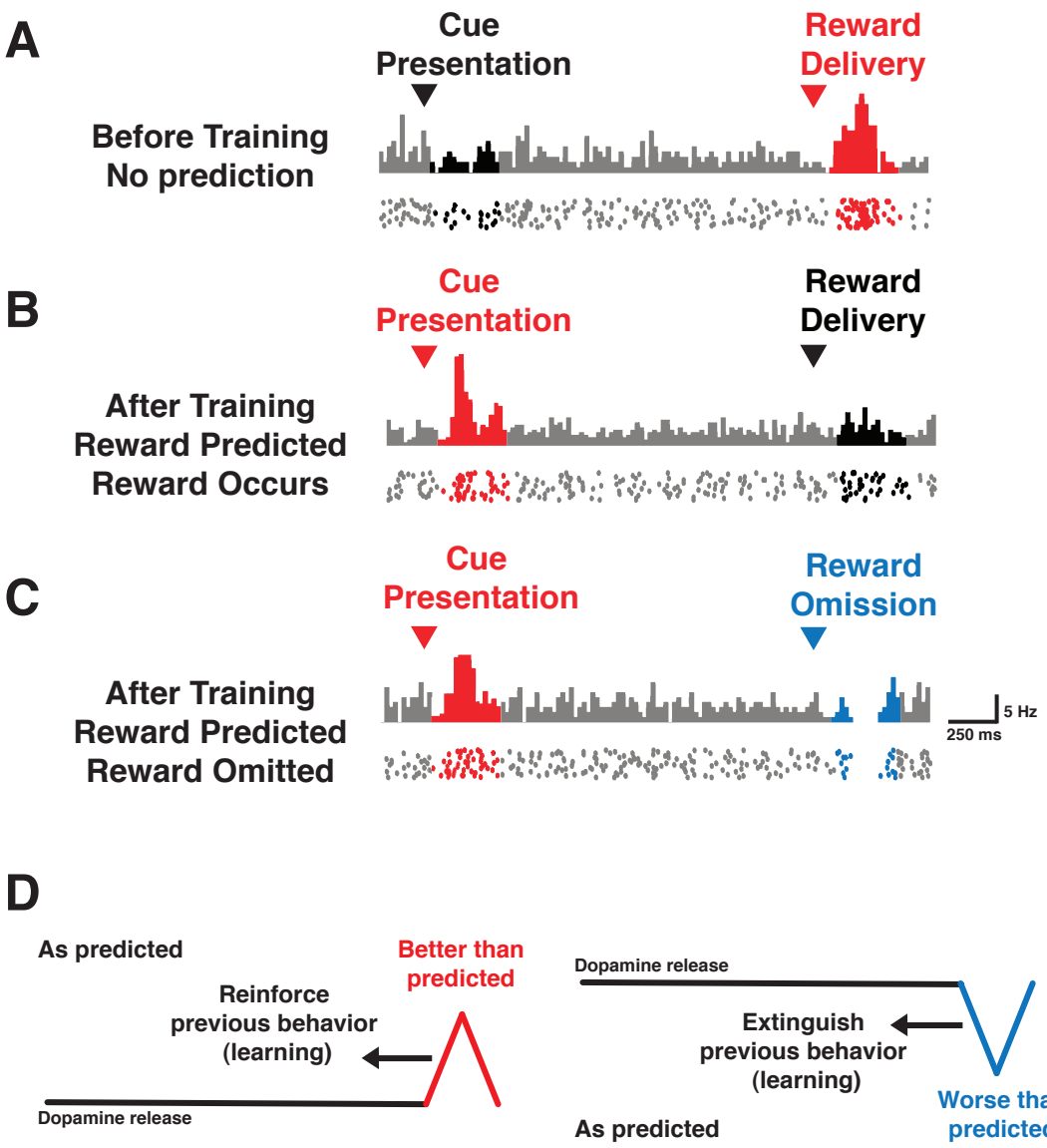
The VTA sends projections to numerous structures throughout the brain, as almost all sources of VTA afferents receive reciprocal projections from the VTA as well (R. D. Oades and G. M. Halliday, 1987). The largest projection from the VTA ascends through the MFB towards a diverse group of forebrain structures (L. W. Swanson, 1982). These include, most significantly, the NAc within the ventral striatum and various prefrontal cortical areas (R. M. Beckstead et al., 1979; L. W. Swanson, 1982). Other targets of this ascending efferent pathway include the amygdala, LH, entorhinal cortex, hippocampus, BNST and septal nuclei (R. M. Beckstead et al., 1979; L. W. Swanson, 1982). A second component of VTA projections ascends dorsally targeting thalamic nuclei, including the LHb (L. W. Swanson, 1982). Additionally, the VTA sends a descending projection that innervates the dorsal and median raphe nuclei, LC, periaquaductal grey and parabrachial nucleus (L. W. Swanson, 1982) (R. M. Beckstead et al., 1979). Interestingly, evidence from retrograde labeling studies suggests that largely separate populations of VTA neurons project to each target region, with only a small degree of overlap (L. W. Swanson, 1982). In line with this finding, more recent studies have found that VTA DA neurons with different projection targets can have distinct electrophysiological characteristics and in some cases anatomical segregation within the VTA (C. P. Ford et al., 2006; S. Lammel et al., 2008).

While a large proportion of VTA efferents are dopaminergic, there is also a significant component of non-dopaminergic VTA projections (L. W. Swanson, 1982). In particular, VTA GABAergic neurons are known to send substantial projections to the PFC and NAc (E. J. Van Bockstaele and V. M. Pickel, 1995; D. B. Carr and S. R. Sesack, 2000). Additionally, *in vivo* studies have found evidence for glutamatergic projections from the VTA to the PFC and NAc (N. Chuhma et al., 2004; A. Lavin et al., 2005), while a recent study utilizing an optogenetic approach has demonstrated the corelease of glutamate from DA terminals in the NAc (G. D. Stuber et al., 2010).

1.2.5 Dopamine neuron reward signals

In vivo single-unit extracellular recordings made from primates during a variety of behavioral tasks have demonstrated that the majority of DA neurons respond to the presentation of primary rewards, such as juice or food, with phasic increases in firing rate (bursts) (W. Schultz, 1998). Interestingly, these reward-evoked responses depend on the unpredictability of the reward (J. Mirenowicz and W. Schultz, 1994). Over the course of behavioral training in which a previously neutral stimulus, such as a light or a tone, is repeatedly paired with the presentation of a reward, DA neurons will gradually stop bursting in response to the reward itself and develop a burst response to the reward-predicting cue (Figure 2A and B) (W. Schultz, 1998). This response is referred to as a conditioned burst response, as the DA neuron has been conditioned through behavioral training to "learn" that this cue reliably predicts the availability of a reward (W. Schultz,

1998). However, when the timing is altered, such that the reward is presented outside of the learned time interval, the DA neuron will again respond with a phasic burst of firing as the reward is once again unexpected (J. Mirenowicz and W. Schultz, 1994). Additionally, DA neuron firing does not only encode positive reward value. After the animal has been trained to learn a specific reward contingency DA neurons show a depression of activity when the reward does not occur at the predicted time (Figure 2C) (W. Schultz, 1998). As a result, phasic DA neuron activity is not thought of simply as a response to rewards, but rather as a signal of reward-prediction error, encoding the difference between the actual reward received and the expected reward (W. Schultz, 1998). Indeed, DA neurons are excited by rewards that are either unexpected or larger than predicted and inhibited by rewards that are either omitted or are smaller than predicted, while rewards that occur exactly as expected do not elicit any change in firing (W. Schultz, 1998). In this manner, increases in DA neuron firing can assign value to stimuli that predict rewards, while reductions in DA neuron firing can decrease value of previously learned stimuli that no longer predict rewards (Figure 2D). Thus, DA release in downstream structures functions as a teaching signal in reward learning.



Adapted from (E. S. Bromberg-Martin et al., 2010)

Figure 2. Dopamine neuron firing encodes a reward prediction error signal.

Firing rate histograms and raster plots from single-unit extracellular *in vivo* recordings of primate DA neurons during behavioral conditioning. (A) At the beginning of training DA neurons burst in response to the unexpected presentation of rewards but do not respond to cue presentation. (B) Following repeated pairings of the cue and reward, the DA neuron becomes conditioned to the cue, bursting in response to the reward-predicting cue and no longer bursting in response to the expected reward. (C) Once the animal has learned that the cue predicts reward delivery omission of the reward at the expected time interval will result in a decrease in DA neuron firing below baseline. As the timing of cue presentation is unexpected, the DA neuron continues to burst in response to the cue presentation throughout training after the cue-reward relationship has been learned. (D) By encoding this reward prediction error signal, DA release functions as a teaching signal in reward learning. Increases in DA reinforce the preceding behavior, whereas decreases in DA release promote extinction of the preceding behavior.

In addition to reward-related responses, a proportion of DA neurons exhibit responses to aversive or noxious stimuli (W. Schultz and R. Romo, 1987; J. Mantz et al., 1989; J. Mirenowicz and W. Schultz, 1996). While the interpretation of these and subsequent findings has been somewhat controversial, recent studies have confirmed that VTA DA neurons can be both excited and inhibited by noxious stimuli and have further suggested that there are distinct populations of DA neurons in the VTA that show differential responses to aversive stimuli (F. Brischoux et al., 2009; M. Matsumoto and O. Hikosaka, 2009). While one population is activated by rewards and inhibited by aversive stimuli, the other is activated by both rewarding and aversive stimuli (O. Valenti et al.; M. Matsumoto and O. Hikosaka, 2009). Finally, there seems to be an anatomical distribution to these two populations with the neurons inhibited by aversive stimuli located more medial and those excited by aversive stimuli located more lateral within the VTA (O. Valenti et al.; M. Matsumoto and O. Hikosaka, 2009). It has been suggested that DA neurons displaying a burst response to both rewarding and aversive stimuli are encoding motivational salience, enabling the animal to act in response to a stimulus of high importance regardless of value (E. S. Bromberg-Martin et al., 2010).

1.2.6 Role of phasic dopamine in downstream structures

As previously noted, the differential patterns of DA release in downstream targets elicited by tonic and phasic firing modes are thought to serve distinct functional roles (W. Schultz, 2007). Electrochemical studies have shown that there is a nonlinear relationship

between DA neuron firing rate and DA release, such that burst firing is particularly efficient at releasing DA (F. G. Gonon, 1988; K. Chergui et al., 1994a). Stimulation of dopaminergic fibers in patterns that simulate burst firing produces significantly greater concentrations of extracellular DA than an equal number of evenly spaced stimulations with the same mean rate (F. G. Gonon, 1988). This difference is due to the inability of the DA reuptake transporter to rapidly clear the DA released by high frequency phasic stimulation from the extrasynaptic space (K. Chergui et al., 1994a).

In vivo studies have shown that the subsecond fluctuations in DA concentration seen in the NAc in response to reward-predicting cues are dependent on burst firing in the VTA and the resulting synaptic overflow of DA (L. A. Sombers et al., 2009). These burst firing-induced DA transients are thought to encode time-specific information and influence learning related to rewards and reward-predicting cues by influencing synaptic plasticity in target structures (R. A. Wise, 2004; W. Schultz, 2007). Consistent with this idea, recent studies have demonstrated a critical role for burst firing in reward learning. For example, selectively attenuating burst firing through genetic inactivation of NMDA receptors in DA neurons results in a significant disruption in the development of cue-dependent reward learning as well as the acquisition of multiple conditioned behaviors, despite the fact that tonic DA neuron firing is unaffected (L. S. Zweifel et al., 2009). In addition, another study found that phasic activation of VTA DA neurons alone, achieved using optogenetic techniques, is sufficient to induce behavioral conditioning (H. C. Tsai et al., 2009). Stimulation in a bursting pattern produced both large DA transients in the NAc and robust CPP while the same number of stimulations delivered in a pattern

simulating tonic firing did not (H. C. Tsai et al., 2009). Taken together, these studies support the concept that phasic DA release acts as a teaching signal by facilitating plasticity in downstream target structures where it functions to "stamp in" reward-related associations, thereby assigning motivational importance to previously neutral stimuli (S. E. Hyman and R. C. Malenka, 2001; R. A. Wise, 2004; S. E. Hyman et al., 2006; W. Schultz, 2007).

1.3 ETHANOL AND THE DOPAMINE SYSTEM

Alcoholism is one of the most prevalent substance abuse disorders worldwide. As such, ethanol abuse has a tremendous negative impact on society, attributable in almost 4% of all deaths globally (J. Rehm et al., 2009). Consequently, a great deal of research has been devoted to identifying the neural mechanisms that contribute to the development of alcoholism. Similarly to other drugs of abuse, many of the reinforcing effects of ethanol appear to be mediated by its actions on the mesolimbic DA system (R. A. Gonzales et al., 2004). Behavioral studies have shown that animals will readily self-administer ethanol directly into the VTA (G. J. Gatto et al., 1994; Z. A. Rodd-Henricks et al., 2000), and that this behavior is dependent on activation of DA neurons (Z. A. Rodd et al., 2004). Further evidence comes from work demonstrating that manipulations of the mesolimbic DA system can alter ethanol-related behaviors (R. A. Gonzales et al., 2004). For example, a wide variety of dopaminergic drugs have been shown to affect ethanol drinking and reinforcement (A. O. Pfeffer and H. H. Samson, 1985, 1988). Blocking

dopaminergic transmission with either microinjections of DA receptor antagonists specifically in the NAc (S. Rassnick et al., 1992; C. W. Hodge et al., 1997) or by destruction of dopaminergic terminals in the NAc with injection of 6-hydroxydopamine attenuates ethanol self-admistration (S. Rassnick et al., 1993). Inactivation of the VTA can also interfere with ethanol reinforcement and intake. Infusion of the D_2 agonist quinpirole into the VTA inhibits DA neuron firing and reduces ethanol-reinforced responses and ethanol-seeking behavior in rats previously trained to self-administer ethanol (C. W. Hodge et al., 1993; S. R. Hauser et al., 2010). Furthermore, studies using ethanol-induced CPP as a measure of ethanol-associated cue learning have found that either inhibition of the VTA through infusion of the $GABA_B$ agonist baclofen or lesioning of the NAc interferes with the acquisition or expression of this behavior, respectively (A. J. Bechtholt and C. L. Cunningham, 2005; C. M. Gremel and C. L. Cunningham, 2008). Together, these data clearly demonstrate that the mesolimbic DA system plays a central role in mediating ethanol-related behaviors.

1.3.1 Acute effects of ethanol on dopamine neurons

In vivo, acute ethanol administration stimulates DA neuron firing in a dose-dependent manner (G. Mereu et al., 1984; G. L. Gessa et al., 1985; M. Foddai et al., 2004). The net effect on firing rate reflects an increase in both spontaneous single-spike firing and burst firing (G. Mereu et al., 1984; M. Foddai et al., 2004). As would be expected, *in vivo* ethanol treatment or self-administration also produces an increase in DA levels measured in the NAc (G. Di Chiara and A. Imperato, 1988; F. Weiss et al.,

1993). Studies using fast-scan cyclic voltammetry have found that, in addition to slow increases in NAc DA levels elicited by ethanol over the course of minutes, ethanol also increases the frequency of subsecond DA transients in the NAc, consistent with the idea that ethanol increases both single spike and burst firing in DA neurons (J. F. Cheer et al., 2007).

The stimulatory effects of ethanol on single spike activity can be detected in the *in vitro* slice preparation (M. S. Brodie et al., 1990; T. Okamoto et al., 2006). As most afferents are severed in the brain slice preparation, this finding suggests that the excitatory effect of ethanol on DA neurons is at least partly due to actions of ethanol on the intrinsic pacemaking mechanisms of DA neurons. Indeed, ethanol increases spontaneous DA neuron firing even in an acutely dissociated preparation (M. S. Brodie et al., 1999). *In vitro* studies using the brain slice preparation to examine the specific ion channels involved in this effect have demonstrated that acute ethanol enhances I_h, a conductance known to contribute to pacemaking in DA neurons, such that in the presence of an I_h antagonist the effect of ethanol on firing rate is greatly reduced (T. Okamoto et al., 2006). In addition to I_h, evidence suggests that ethanol may modulate other ion channels known to be involved in pacemaking in DA neurons, including the A-type and M-type K+ channels (H. Morikawa and R. A. Morrisett, 2010). However, to date these channels have not been shown to play a major role in the excitatory effect of ethanol on VTA DA neurons (H. Morikawa and R. A. Morrisett, 2010).

As DA neuron firing activity *in vivo* is mainly regulated by synaptic inputs (A. A. Grace et al., 2007), modulation of these inputs likely contributes to the excitatory effects

of ethanol. In particular, a diverse array of glutamatergic inputs are prominently involved in regulating DA neuron firing (S. Geisler and R. A. Wise, 2008). Interestingly, ethanol does not appear to directly stimulate DA neuron firing through actions on glutamate receptors. No acute effects of ethanol on AMPA receptor-mediated currents have been reported, while multiple studies have demonstrated that acute ethanol in fact, inhibits NMDA receptor function (D. M. Lovinger et al., 1989, 1990). However, ethanol can indirectly facilitate glutamatergic transmission in the VTA through presynaptic actions on glutamatergic terminals (C. Deng et al., 2009). In this study, ethanol was found to enhance AMPA-mediated transmission and increase levels of glutamate in the VTA in a manner dependent on local release of DA and activation of D1 receptors. Accordingly, D1 antagonists significantly reduced, but did not abolish, the excitatory effect of ethanol on DA neuron firing. These findings suggest that ethanol initiates a positive feedback loop, whereby DA release within the VTA facilitates glutamatergic transmission, which further stimulates DA release.

Another major regulatory influence on VTA DA neuron firing comes from GABAergic afferents known to maintain a tonic inhibition on DA neuron activity (S. W. Johnson and R. A. North, 1992a; S. C. Steffensen et al., 1998). Activation of these inputs inhibits both single-spike and burst firing in DA neurons (H. R. Olpe et al., 1977; V. Seutin et al., 1994; S. Erhardt et al., 2002), while inhibition of GABAergic neurons in the VTA enhances DA neuron firing (J. H. Ye et al., 2004). These findings suggest that the stimulatory effects of ethanol could be due to disinhibition of DA neuron firing through actions on GABAergic inputs. Indeed, *in vivo* ethanol administration dose-dependently

inhibits VTA GABA neuron firing (R. A. Gallegos et al., 1999), an effect that appears to be primarily due to inhibition of NMDA-mediated excitation of GABAergic neurons within the VTA (S. H. Stobbs et al., 2004). Similarly, an *in vitro* study also found that acute ethanol exposure potently inhibits VTA GABA neuron firing (C. Xiao et al., 2007). Importantly, this study also found that the ethanol-induced decrease in GABA activity was accompanied by increased DA neuron firing, an effect that could be blocked by the $GABA_A$ antagonist bicuculline or significantly reduced by DAMGO, a μ–opioid agonist known to inhibit GABA neuron firing in the VTA (K. Gysling and R. Y. Wang, 1983). Additionally, a behavioral study has shown that microinjections of $GABA_A$ antagonists in the VTA decrease ethanol consumption without affecting saccharin consumption (K. L. Nowak et al., 1998). These findings support the idea that disinhibition of DA neuron firing via inhibition of GABAergic inputs contributes to the stimulatory effects of ethanol.

Due to numerous similarities between the acute effects and withdrawal symptoms associated with abuse of ethanol and opiate drugs, the role of the opioid system in mediating the effects of ethanol has also been extensively studied (A. Herz, 1997). For example, it has been known for some time that systemic injection of μ-opioid receptor antagonists, such as naloxone and naltrexone, causes a dose-dependent decrease in ethanol consumption in multiple animal models (H. L. Altshuler et al., 1980; R. D. Myers et al., 1986; J. C. Froehlich et al., 1990). More recently, studies have focused specifically on the role of opioid receptor signaling in the VTA in mediating ethanol reward. Acute ethanol exposure causes release of β-endorphin in the VTA (S. Jarjour et al., 2009),

where μ-opioid receptor activation is known to increase DA neuron firing via disinhibition (K. Gysling and R. Y. Wang, 1983; S. W. Johnson and R. A. North, 1992b). I*n vitro* brain slice experiments have shown that this opioid receptor-mediated disinhibition may play a role in mediating the effects of ethanol on DA neuron firing, as the opioid receptor antagonist naloxone attenuates the ethanol-induced excitation of DA neurons (C. Xiao et al., 2007). Findings from behavioral experiments have been consistent with these studies, as systemic injection of naltrexone inhibits the increase in NAc DA levels occurring during ethanol self-administration (R. A. Gonzales and F. Weiss, 1998), while microinjection of the μ-opioid receptor antagonist, nalmefene, directly into the VTA suppresses oral ethanol self-administration (H. L. June et al., 2004). Currently, multiple opioid receptor antagonists are in use or being considered for use in treatment of alcoholic patients, as these drugs have shown some efficacy in reducing ethanol consumption and craving in humans (S. S. O'Malley, 1996; M. Soyka and S. Rosner, 2008).

In addition to glutamate, GABA and the opioids, both serotonin and acetylcholine seem to play an important role in enabling ethanol to stimulate DA neuron activity. Interestingly, in vitro slice recordings have shown that perfusion of either nicotine or serotonin significantly enhances the stimulatory effect of ethanol on DA neuron firing, even at concentrations that have no effect in the absence of ethanol (M. S. Brodie et al., 1995; A. Clark and H. J. Little, 2004). Consistent with these findings, intra-VTA injection of serotonin or nACh receptor antagonists inhibit DA release in the NAc following ethanol administration (O. Blomqvist et al., 1997; Q. S. Yan et al., 2005).

Behavioral studies further support a role for serotonin and acetylcholine in the reinforcing effects of ethanol, as both systemic and intra-VTA injections of serotonin and nACh receptor antagonists decrease ethanol consumption in rodents (F. Fadda et al., 1991; O. Blomqvist et al., 1996; J. M. Farook et al., 2009; L. M. Hendrickson et al., 2009; A. Kuzmin et al., 2009). In fact, both serotonergic and cholinergic drugs are currently under investigation as potential treatments for alcoholism in humans (S. A. McKee et al., 2009; G. A. Kenna, 2010).

1.3.2 Adaptive responses of dopamine neurons to ethanol exposure

The transition from casual alcohol use to dependence and addiction is thought to depend on neuroadaptations that develop over time in response to chronic alcohol exposure (M. Melis et al., 2005). Thus, elucidating the plastic changes within the mesolimbic DA system induced by repeated or chronic ethanol exposure is of critical importance. *In vivo* recordings in brain slices prepared from animals repeatedly exposed to ethanol have shown that while VTA DA neurons exhibit an increased response to ethanol as compared to saline-treated controls (M. Diana et al., 1992), withdrawal from a repeated ethanol treatment regimen (2-5 g/kg intragastrically, every 6 hours for 6 days) reveals a significant reduction in DA neuron activity (M. Diana et al., 1993). More specifically, spontaneous firing, spikes per burst and the overall number of bursts are all reduced following withdrawal from ethanol treatment (M. Diana et al., 1993). These decreases in dopaminergic activity persist for at least 72 hrs following the last ethanol exposure (M. Diana et al., 1996). These reductions in DA neuron activity are paralleled

by dramatic decreases in DA concentrations measured in the NAc and can be reversed with an acute administration of ethanol (Z. L. Rossetti et al., 1992; M. Diana et al., 1993). In addition, human studies have also demonstrated a reduction in NAc DA release or DA transmission in detoxified alcoholics (D. Martinez et al., 2005; N. D. Volkow et al., 2007).

This hypodopaminergic state is hypothesized to contribute to the anhedonia and dysphoria associated with ethanol withdrawal (M. Melis et al., 2005). In line with this, animals experiencing withdrawal from a chronic 3-5 week ethanol treatment will self-administer ethanol at a level that restores NAc DA to prewithdrawal levels (F. Weiss et al., 1996). In addition, studies using intracranial self-stimulation thresholds as a measure of affective motivational state have shown that during withdrawal from chronic ethanol exposure (17-20 days in ethanol vapor chambers) animals require a higher stimulation intensity to maintain intracranial self-stimulation (G. Schulteis et al., 1995). This decreased sensitivity of the reward system is thought to be a key feature driving drug-seeking behavior in addiction (G. F. Koob et al., 1998). Consistent with this idea, human imaging studies in detoxified alcoholics have found a correlation between decreased accumbal responses related to non-drug rewards and intensity of alcohol cravings in response to alcohol-related cues (J. Wrase et al., 2007).

As with acute ethanol, withdrawal from chronic ethanol affects both burst and spontaneous firing, raising the possibility that chronic exposure to ethanol may be affecting both intrinsic firing and neurotransmitter inputs onto DA neurons. Studies focusing on mechanisms underlying intrinsic firing have reported changes in some ionic

conductances involved in DA neuron pacemaking following ethanol exposure. A study by Okamoto et al. found that I_h density was significantly decreased in VTA DA neurons following 1 day of withdrawal from 5 day ethanol treatment (2 g/kg, i.p., once daily)(T. Okamoto et al., 2006), an effect that was also seen following 7 days of withdrawal from 5 day ethanol treatment (2 g/kg, i.p., twice daily) in another study (F. W. Hopf et al., 2007). Additionally, the Hopf et al study found a decrease in the function of SK channels following 7 days of withdrawal (F. W. Hopf et al., 2007). However, these and multiple other *in vitro* studies have failed to find any difference in baseline rate of spontaneous firing following withdrawal from repeated *in vivo* ethanol exposure (ranging from 2-3.5 g/kg, i.p., 5-21 days)(M. S. Brodie, 2002; T. Okamoto et al., 2006; F. W. Hopf et al., 2007; S. Perra et al., 2011), consistent with prior studies suggesting that I_h and SK are not major regulators of pacemaking in VTA DA neurons (J. Wolfart et al., 2001; Z. M. Khaliq and B. P. Bean, 2010). These findings suggest that the hypodopaminergic state that follows withdrawal from ethanol exposure may not result from alterations in intrinsic pacemaking. However, it is important to note that one group has found a reduction in baseline firing of DA neurons following very high levels of chronic ethanol exposure (25-32 g/kg/day, 21-23 days), though the mechanism underlying this effect was not identified (C. P. Bailey et al., 1998; C. P. Bailey et al., 2001).

Studies examining the effects of ethanol on GABAergic transmission within the VTA have proposed that adaptations in this system may contribute to decreased DA neuron activity during ethanol withdrawal. *In vivo* recordings have shown that following withdrawal from 2 weeks of chronic ethanol exposure, VTA GABA neurons have

significantly increased baseline firing rates and develop tolerance to the acute inhibitory effects of ethanol (R. A. Gallegos et al., 1999). In addition, following a single ethanol exposure (2 g/kg i.p.) there is an increase in both spontaneous and evoked GABA release onto VTA DA neurons recorded in brain slices (M. Melis et al., 2002; M. J. Wanat et al., 2009). This effect lasts at least a week and is mediated by activation of the cAMP-PKA signaling cascade leading to increased probability of release at GABAergic terminals (M. Melis et al., 2002). However, another study using a longer, chronic ethanol exposure (3.5 g/kg i.p, twice daily for at least 21 days) found a significant reduction in inhibition of DA neuron firing induced by perfusion of GABA suggesting that neuroadaptations within GABAergic neurons of the VTA may vary dramatically depending on the ethanol exposure regimen (M. S. Brodie, 2002).

The mechanisms involved in feedback inhibition of VTA DA neurons via local somatodendritic DA release represent another potential target through which chronic ethanol could modulate the activity of the mesolimbic DA system. In a recent study, electrophysiological recordings in brain slices were used to demonstrate that D_2 receptor/GIRK-mediated inhibition of DA neurons is significantly enhanced following withdrawal from repeated in vivo ethanol exposure (2 g/kg, i.p., 3 times daily for 7 days), an effect resulting from a decrease in intracellular Ca^{2+}-dependent desensitization of D_2 receptors (S. Perra et al., 2011). Such an enhancement of D2 autoinhibition may contribute to the hypodopaminergic state that develops during ethanol withdrawal.

In addition to changes in the mechanisms of autoinhibition, other studies have identified alterations in DA neuron terminals affecting release in the target structures.

Studies in self-administering rats and monkeys have demonstrated that chronic ethanol exposure (8 weeks – 18 months) leads to an increase in the rate of dopamine uptake in the striatum (E. A. Budygin et al., 2003; M. R. Carroll et al., 2006). While these studies do not discern whether this change is the result of increased dopamine transporter (DAT) density or function, other studies have found increases in NAc DAT binding sites in rodents (Y. Itzhak and J. L. Martin, 1999; X. Jiao et al., 2006) and in DAT number in monkeys (D. C. Mash et al., 1996) following withdrawal from chronic ethanol exposure. In any of these cases, increased DA uptake may also contribute to the decreased DA levels found in NAc during ethanol withdrawal.

Numerous studies examining ethanol-induced adaptations in glutamatergic transmission in VTA DA neurons have produced complex and sometimes conflicting results. Biochemical analysis of VTA tissue homogenates found that chronic ethanol exposure (12 weeks) causes an increase in the expression of the GluR1 and GluR2 subunits of the AMPA receptor (J. Ortiz et al., 1995). Consistent with these findings, ethanol exposure enhances AMPA-receptor mediated transmission onto DA neurons as measured in a brain slice preparation, either following a single injection (2 g/kg i.p.) in C57BL/6 mice (D. Saal et al., 2003), or following chronic voluntary ethanol drinking (35-50 days) in rats (G. D. Stuber et al., 2008). This enhancement of excitatory transmission is also triggered by multiple other drugs of abuse, as well as stress, and is most likely due to increased insertion of AMPA receptors into the postsynaptic membrane (M. A. Ungless et al., 2001; D. Saal et al., 2003). As DA neuron activity is clearly decreased during ethanol withdrawal, the immediate functional implications of an

increase in AMPA-mediated transmission are not clear. This global potentiation of excitatory inputs onto DA neurons could promote DA-dependent reward learning by enhancing DA release in target structures in response to future stimuli, however this has not yet been shown experimentally (J. A. Kauer and R. C. Malenka, 2007). It should be noted, however, that a similar study by the same group found a decrease in AMPA transmission in DBA mice 24 hrs after an ethanol injection, (2 g/kg, i.p.), while failing to replicate the increase previously seen in C57 mice. This study only differed in that a lower concentration of ethanol solution was used for injection, though the same dose was given (M. J. Wanat et al., 2009).

Despite the fact that chronic ethanol treatment leads to increased expression of the NR1 subunit of NMDA receptors in VTA (J. Ortiz et al., 1995), NMDA receptor-mediated transmission is not altered in DA neurons in brain slices from rats or C57 mice following a single ethanol injection (2 g/kg, i.p.) (M. J. Wanat et al., 2009), a short period of repeated ethanol injections (2 g/kg, i.p., 2 times daily for 5 days) (F. W. Hopf et al., 2007), or an extended period of repeated ethanol injections (3.5 g/kg, i.p. 2 times daily for > 21 days) (M. S. Brodie, 2002). These findings suggest that NMDA receptor-mediated transmission is not prominently involved in the decrease in DA neuron activity seen in withdrawal. However, similarly to AMPA currents, NMDA currents are reduced in DBA mice following a single injection of ethanol (2 g/kg, i.p.) (M. J. Wanat et al., 2009), though the functional implications of these strain differences are not currently understood.

1.4 LEARNING AND MEMORY MECHANISMS IN ADDICTION

One of the defining aspects of drug addiction is its persistent nature. Addiction in human patients is typically a chronic condition, often relapsing despite repeated treatment attempts and prolonged periods of abstinence (S. E. Hyman et al., 2006). As such, addiction has come to be thought of as a learning and memory disorder, in which the pathological formation of extremely stable, long-term drug-associated memories within reward circuits underlie persistent and uncontrollable drug taking behavior (S. E. Hyman et al., 2006; J. A. Kauer and R. C. Malenka, 2007). The origins of this "pathological learning" theory of addiction arose from studies recognizing similarities between the responses of addicts to drug-associated cues and Pavlovian conditioned responses (A. Wikler, 1948). Future research eventually led to an understanding of the powerful influence of cues on drug craving and use. Cues previously associated with drug use strongly influence relapse, as they can initiate and maintain drug-taking behavior in both humans and animal models (J. Stewart et al., 1984; C. P. O'Brien et al., 1992; D. W. Self, 1998).

The specificity of responses of addicts and laboratory animals to drug cues suggest that both associative and synapse-specific learning mechanisms are involved in addiction (S. E. Hyman and R. C. Malenka, 2001). Thus, work in the addiction field has begun to focus on long-term potentiation (LTP) and long-term depression (LTD), the major candidate cellular mechanisms thought to underlie associative and synapse-specific learning and memory throughout the nervous system. Indeed, recent work has identified

many commonalities in the cellular and molecular mechanisms involved in LTP and LTD (and presumably learning) and addiction (A. E. Kelley, 2004). For example, LTP and LTD are often dependent on NMDA receptor activation (R. C. Malenka and M. F. Bear, 2004). Similarly, behavioral senstization, acquisition of drug self-administration and CPP can all be blocked by pretreatment with NMDA receptor antagonists (S. Schenk et al., 1993; M. Jeziorski et al., 1994; H. S. Kim et al., 1996). In addition, molecular pathways normally involved in triggering the alterations in gene expression and synaptic structure that underlie LTP and LTD, such as the cAMP/PKA/CREB pathway, are activated by exposure to addictive drugs (J. D. Berke and S. E. Hyman, 2000). Clearly, there is strong evidence to support the idea that addictive substances co-opt normal learning processes within reward circuits. Therefore, the continued study of these forms of synaptic plasticity and their modulation by addictive drugs is critical to our understanding of the development and persistence of addiction (J. A. Kauer and R. C. Malenka, 2007).

1.4.1 Ethanol and synaptic plasticity

In light of the extensive evidence suggesting that addiction involves maladaptive "overlearning" of drug-related experiences, it remains somewhat puzzling that ethanol has long been known to impair numerous types of learning and memory in both humans and animals (R. S. Ryback, 1971; A. E. Ryabinin, 1998). For example, acute ethanol intoxication impairs short and long term memory (R. Kalin, 1964; B. M. Jones, 1973; M. E. Miller et al., 1978), passive avoidance learning (G. Bammer and G. B. Chesher, 1982),

both spatial and nonspatial working memory (W. E. Gibson, 1985; C. L. Melchior et al., 1993), fear conditioning (K. R. Melia et al., 1996) and context-dependent drug conditioning (C. L. Cunningham and C. M. Gremel, 2006). Similar effects are seen with chronic ethanol, as decreases in learning capacity have also been reported in long-term alcoholics and in animals withdrawn from repeated ethanol exposure (R. S. Ryback, 1971; A. E. Ryabinin, 1998; D. N. Stephens et al., 2005).

Consistent with its effects on learning and behavior, both acute and chronic ethanol exposures have been shown to suppress activity-dependent synaptic plasticity in multiple brain areas. Acute ethanol inhibits both LTP and LTD in hippocampus, due to a combination of direct inhibition of NMDA receptors and effects on GABAergic transmission (R. A. Morrisett and H. S. Swartzwelder, 1993; Y. Izumi et al., 2005). Ethanol also blocks multiple forms of LTD in the cerebellum through inhibitory effects on voltage-gated Ca^{2+} channels and mGluRs (M. Carta et al., 2006; A. Belmeguenai et al., 2008). Finally, acute and chronic ethanol exposures impair LTP in dorsomedial striatum and corticostriatal LTD, respectively (J. X. Xia et al., 2006; H. H. Yin et al., 2007; G. Q. Xie et al., 2009).

As mentioned previously, ethanol exposure can produce an increase in AMPA receptor-mediated transmission in the VTA (D. Saal et al., 2003; G. D. Stuber et al., 2008). However, as this effect is global and lacks synapse specificity, it seems unlikely to be involved in the cue-specific associative learning seen in addiction. Thus, it currently remains unclear how ethanol exposure promotes the formation of the long-term drug-related memories that characterize alcoholism.

1.4.2 Synaptic plasticity in the VTA

The role of the mesolimbic DA system in both reward learning and addiction is well established. In addition, findings from numerous studies suggest that the cellular mechanisms involved in LTP and LTD in other brain regions are involved in drug-induced behaviors in the VTA (J. A. Kauer and R. C. Malenka, 2007). Throughout the CNS, different forms of synaptic plasticity are dependent on NMDA receptor activation (R. C. Malenka and M. F. Bear, 2004), while, within the VTA, interfering with NMDA receptors can inhibit drug-related behaviors including CPP and behavioral sensitization (P. W. Kalivas and J. E. Alesdatter, 1993; G. C. Harris et al., 2004; L. S. Zweifel et al., 2008). These findings further support the idea that LTP and LTD mechanisms in the VTA may be involved in drug-related behaviors. Therefore, identifying and understanding the mechanisms underlying synaptic plasticity within this key component of the reward circuit is of critical importance to the study of addiction.

1.4.2.1 Plasticity of AMPA receptor-mediated transmission

Early studies pairing high frequency pre-synaptic stimulation with post-synaptic depolarizations, known to reliably induce LTP in hippocampal pyramidal neurons, demonstrated that glutamatergic synapses onto DA neurons in both the VTA and SNc could express LTP (A. Bonci and R. C. Malenka, 1999; P. G. Overton et al., 1999; H. D. Mansvelder and D. S. McGehee, 2000). As in the hippocampus, this form of high

frequency stimulation LTP is dependent on NMDAR activation (A. Bonci and R. C. Malenka, 1999). As described previously, drug exposure can trigger a global plasticity of AMPA-mediated transmission in DA neurons that is also dependent on NMDAR activation and is mediated by increased trafficking of AMPA receptors to the postsynaptic membrane (M. A. Ungless et al., 2001). Induction of this drug-induced plasticity occludes synaptically evoked high frequency stimulation LTP, suggesting increased AMPA receptor trafficking may underlie this LTP as well (M. A. Ungless et al., 2001; J. A. Kauer and R. C. Malenka, 2007). Interestingly, another study found that LTP of AMPA receptor-mediated transmission can also be induced in VTA DA neurons when eliminating the presynaptic stimulation and instead pairing postsynaptic depolarization with bath application of nicotine, which activates NMDA receptors by enhancing glutamate release through actions on presynaptic α7 subunit-containing nACh receptors (H. D. Mansvelder and D. S. McGehee, 2000). This study clearly demonstrates the potential for addictive drugs to powerfully modulate the cellular correlates of learning and memory within reward circuits.

More recently, another group has described a spike-timing dependent form of LTP in VTA DA neurons induced with a protocol that paired bursts of presynaptic stimulation with bursts of postsynaptic spikes. (Q. S. Liu et al., 2005). In this study, spike-timing dependent LTP was generated by evoking 20 burst pairs with an interval of 100 ms in which the onset of presynaptic stimulations preceded the postsynaptic spike by 5 ms. Unlike the high frequency stimulation LTP described previously, which is occluded by a single cocaine injection (M. A. Ungless et al., 2001), spike-timing

dependent LTP could only be evoked in slices from animals that received 5-7 days of cocaine injections (15 mg/kg once daily) and not in slices from saline-treated controls. Furthermore, this LTP depends on a cocaine-induced reduction in GABA-mediated inhibition of DA neurons, as it can be blocked by application of benzodiazepenes and can be induced in saline-treated animals by running the pairing protocol in the presence of $GABA_A$ antagonists (Q. S. Liu et al., 2005). Drug-induced reductions in GABAergic tone could therefore play an important role in facilitating long-term synaptic modifications within the VTA that contribute to drug-seeking behavior or the formation of drug associations (Q. S. Liu et al., 2005). This role for GABAergic inhibition in gating LTP in DA neurons is consistent with findings in hippocampus where suppression of GABAergic inhibition also facilitates LTP induction (H. Wigstrom and B. Gustafsson, 1983; R. M. Meredith et al., 2003). It is important to note however, that another group examining spike-timing dependent LTP evoked with an identical protocol found that a single injection of cocaine (15 mg/kg), given 24 hours prior to recording, completely occluded expression of LTP, similarly to high frequency stimulation LTP (P. Luu and R. C. Malenka, 2008). In addition, this study found that spike-timing dependent LTP depends upon rises in postsynaptic Ca^{2+} and PKC.

In addition to LTP, two different forms of AMPA LTD are expressed at excitatory synapses on VTA DA neurons. The initial report of LTD in the VTA described a form of plasticity induced by pairing low frequency stimulation of excitatory inputs (1 Hz for 6 min) with depolarization of the postsynaptic neuron to -40 mV (S. Jones et al., 2000). Unusually, the LTD produced by this low frequency stimulation pairing is not dependent

on NMDA receptor activation, as is LTD in many other brain areas. However, this form of plasticity does depend on Ca^{2+} entry through voltage-gated Ca^{2+} channels and can be recapitulated without presynaptic stimulation simply by triggering Ca^{2+} entry with repeated depolarization of the postsynaptic neuron (S. Jones et al., 2000). This study also reported that application of amphetamine to the slice prior to pairing blocks induction of LTD, which was the first demonstrated effect of an addictive drug on synaptic plasticity in the VTA (S. Jones et al., 2000).

In a later study, Bellone and Luscher described a distinct form of LTD of excitatory inputs onto DA neurons that is dependent on activation of mGluRs (C. Bellone and C. Luscher, 2005). This LTD is induced by applying brief bursts of high frequency presynaptic stimulation (2 trains of 5 stimuli at 66 Hz) and is blocked by mGluR antagonists. Mechanistically, mGluR LTD is mediated by the switching of GluR2 subunit-lacking AMPA receptors for GluR2-containing AMPA receptors. Furthermore, this mGluR LTD can also be produced simply by perfusing the mGluR agonist DHPG. Interestingly, both low frequency stimulation-induced LTD and mGluR-dependent LTD coexist in the same synapses and the induction of one does not occlude the subsequent induction of the other (C. Bellone and C. Luscher, 2005).

1.4.2.2 Plasticity of NMDA receptor-mediated transmission

Despite the fact that NMDA receptor activation plays a predominant role in generating the DA neuron burst firing thought to function as a teaching signal in reward learning, relatively few studies have examined synaptic plasticity of NMDA receptor-

mediated transmission onto DA neurons (W. Schultz, 1998; L. S. Zweifel et al., 2008; L. S. Zweifel et al., 2009).

A small number of *in vitro* studies have shown that neuropeptides can modulate NMDA receptor-mediated transmission in VTA DA neurons. Bath perfusion of CRF, a stress-related peptide, or orexin, an arousal and feeding-related peptide, can produce a transient and global potentiation of NMDA receptor currents in brain slices (M. A. Ungless et al., 2003; S. L. Borgland et al., 2006). Both orexin and CRF releasing neurons project to the VTA from the LH and BNST, respectively, where they synapse on DA neurons (J. J. Balcita-Pedicino and S. R. Sesack, 2007; P. Tagliaferro and M. Morales, 2008). Interestingly, both peptides exert these effects through activation of a PLC/PKC-dependent signaling pathway. Rather than underlying synapse-specific learning itself, the transient and global nature of this potentiation suggests it may function to enhance other forms of NMDAR-dependent LTP (A. Bonci and S. Borgland, 2009).

Recently, a study has reported the first demonstration of synaptic activity-dependent LTP of NMDAR-mediated transmission onto DA neurons (M. T. Harnett et al., 2009). This NMDAR LTP is posited to contribute to the development of the conditioned-burst response seen in *in vivo* recordings of DA neurons during Pavlovian conditioning (W. Schultz, 1998; M. T. Harnett et al., 2009). In line with this idea, LTP is induced with a pattern of stimulation thought to mimic the activity experienced by the DA neuron during behavioral conditioning. More specifically, a sustained stimulation of excitatory presynaptic inputs (70 stimuli at 50 Hz, meant to represent the activity induced by presentation of a cue) is paired with a burst of action potentials in the DA neuron (5

APs at 20 Hz, mimicking the burst response to reward presentation) (Figure 3). Repeatedly subjecting the DA neuron to this pairing (10x) produces LTP that is dependent on activation of NMDA receptors and mGluRs, as well as the timing of the burst in relation to presynaptic stimulation. Furthermore, this study demonstrated that the critical signal for induction of this burst-timing dependent LTP is facilitation of AP-associated Ca^{2+} signals by mGluR and IP_3-mediated release of Ca^{2+} from intracellular stores (Figure 3) (M. T. Harnett et al., 2009). This finding is consistent with the involvement of postsynaptic AP-associated Ca^{2+} signals and mGluR activation in plasticity in hippocampus and cortex (Z. A. Bortolotto et al., 1999; C. R. Rose and A. Konnerth, 2001; P. J. Sjostrom and S. B. Nelson, 2002; T. Nevian and B. Sakmann, 2006) and highlights the potential for modulation of synaptic plasticity in the VTA through manipulations affecting Ca^{2+} signaling.

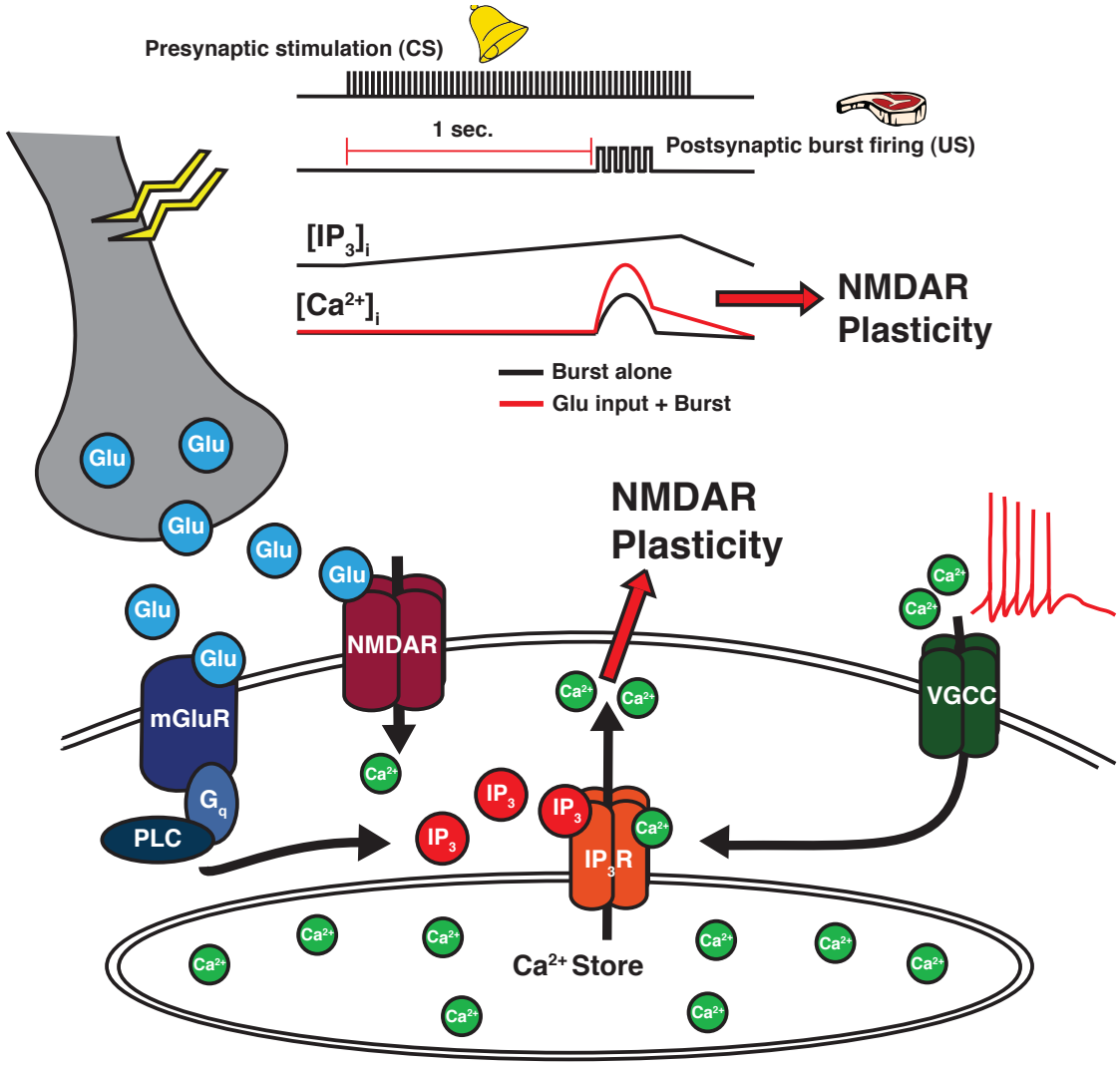


Figure 3. Burst-timing dependent LTP of NMDAR-mediated transmission in DA neurons.

Repeatedly pairing presynaptic stimulation of glutamatergic inputs and postsynaptic bursts of APs induces LTP of NMDAR-mediated transmission. mGluR-mediated production of IP_3 slowly increases during the presynaptic stimulation train and facilitates the burst-associated Ca^{2+} signal, triggering induction of LTP.

1.5 CALCIUM SIGNALING IN DOPAMINE NEURONS

Throughout the nervous system intracellular Ca^{2+} signaling is involved in the regulation of many cellular processes including excitability, transmitter release, gene transcription and synaptic plasticity (M. J. Berridge, 1998). In particular, rises in intracellular Ca^{2+} concentrations in response to AP firing or synaptic inputs commonly function as induction signals in synaptic plasticity in a variety of brain regions (C. R. Rose and A. Konnerth, 2001; P. J. Sjostrom and S. B. Nelson, 2002). In DA neurons, AP firing triggers Ca^{2+} influx through activation of voltage-gated Ca^{2+} channels (VGGCs), which then activates the small-conductance Ca^{2+}-activated K^+ channel (SK) (Figure 4) (J. Wolfart and J. Roeper, 2002; G. Cui et al., 2007). In addition, synaptic activation of G_q-coupled receptors, such as mGluRs, leads to the production of IP_3 and release of Ca^{2+} from intracellular stores through IP_3 receptors, resulting in a slow hyperpolarization mediated by SK channel activation (C. D. Fiorillo and J. T. Williams, 1998; H. Morikawa et al., 2000). In both cases, intracellular Ca^{2+} can then elicit further Ca^{2+} release from stores through IP_3 and ryanodine (RyR) receptors in a regenerative process known as Ca^{2+}-induced Ca^{2+} release (CICR) (Figure 4) (M. J. Berridge, 1998; G. Cui et al., 2007). Through CICR, focal activation of mGluRs can initiate a Ca^{2+} wave that spreads through both the soma and dendrites (H. Morikawa et al., 2003). Functionally, AP-induced SK activation is thought to contribute to the afterhyperpolarization (AHP), thereby modulating firing patterns and frequency, though to a lesser extent in VTA DA neurons as compared to those in SNc (J. Wolfart et al., 2001; G. Cui et al., 2007). The mGluR-

mediated hyperpolarization, on the other hand, is thought to underlie the pauses in firing that frequently follow bursts and may, therefore, play a critical role in regulating DA neuron excitability by setting the frequency with which bursts can occur (H. Morikawa et al., 2003).

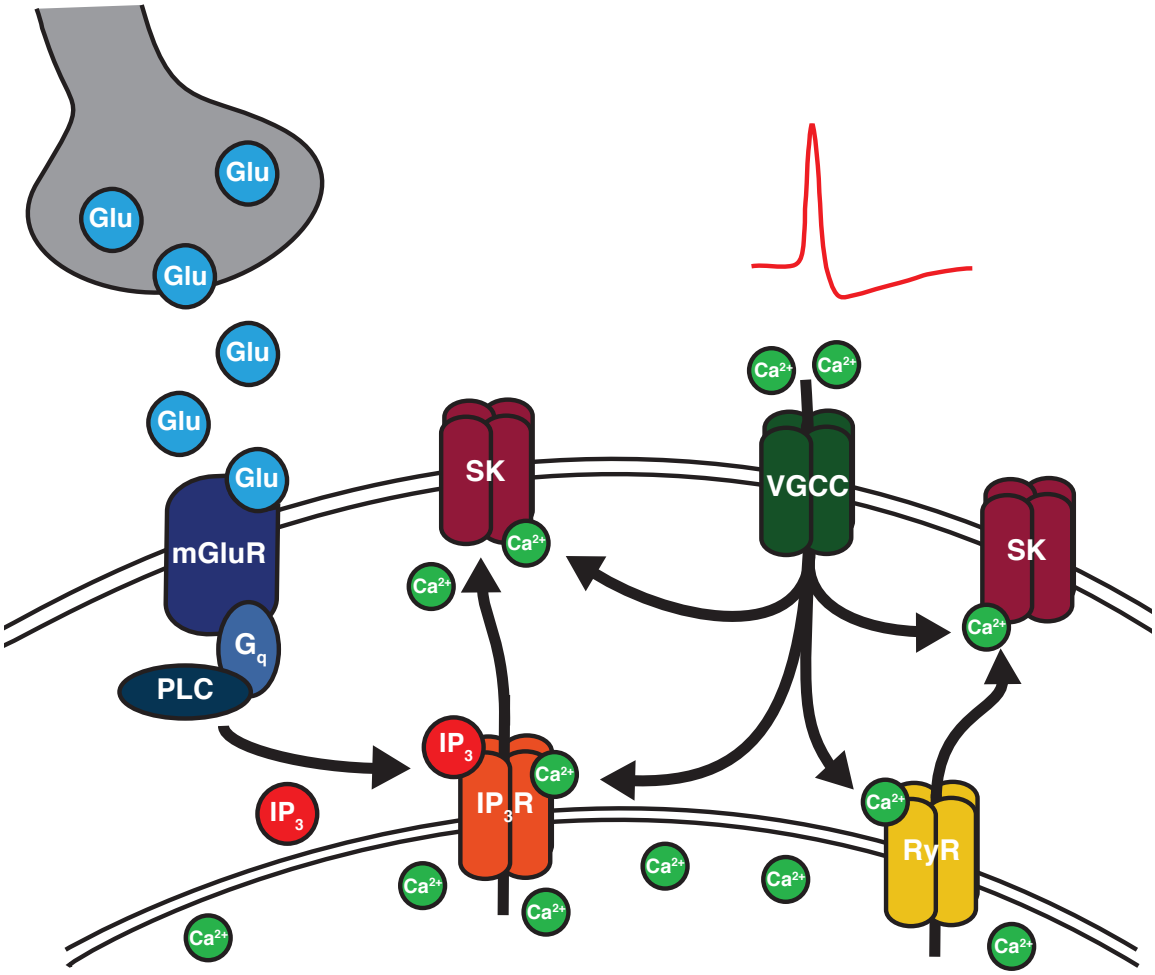


Figure 4. Ca^{2+} signaling pathways in DA neurons.

Extracellular Ca^{2+} enters the cell through VGCCs during AP firing where it can directly activate SK channels or contribute to CICR from intracellular stores through activation of IP_3 and RyR receptors. Activation of mGluRs leads to the production of IP_3 and release of Ca^{2+} from intracellular stores through IP_3Rs. As they are coactivated by Ca^{2+} and IP_3, IP_3Rs can act as coincident detectors between AP firing and glutamatergic synaptic inputs.

1.5.1 IP_3 receptors as coincidence detectors

IP_3 receptors are tetrameric Ca^{2+} channels found on the endoplasmic reticulum of both excitable and non-excitable cells throughout the body (C. W. Taylor and A. J. Laude, 2002). Interestingly, simultaneous binding of both IP_3 and Ca^{2+} are required for channel opening (C. W. Taylor and A. J. Laude, 2002). IP_3 receptor gating is complex, as in addition to the IP_3 binding site, IP_3 receptor subunits contain both stimulatory and inhibitory Ca^{2+} binding sites with differing Ca^{2+} binding affinities (C. W. Taylor and A. J. Laude, 2002). Coactivation by IP_3 and Ca^{2+} is thought to derive from coordination between the three binding sites such that IP_3 binding shifts the affinity of the two Ca^{2+} binding sites in favor of the stimulatory site (C. W. Taylor and A. J. Laude, 2002). This property allows the IP_3 receptor to function as a coincident detector in neurons, activating when AP firing-induced Ca^{2+} influx occurs coincident with synaptic inputs to metabotropic receptors generating IP_3 production (Figure 4). Indeed, when synaptic or pharmacological activation of G_q-coupled receptors is paired with AP firing in DA neurons there is a robust facilitation of the intracellular Ca^{2+} signal (G. Cui et al., 2007; M. T. Harnett et al., 2009). As described previously, this facilitation of AP-associated Ca^{2+} signals functions as the induction signal for LTP of NMDA receptor-mediated transmission in DA neurons (Figure 3) (M. T. Harnett et al., 2009).

1.5.2 Ethanol and calcium signaling

While the effects of ethanol exposure on calcium signaling in DA neurons are currently unknown, many studies have shown that IP_3-mediated Ca^{2+} mobilization is sensitive to both acute and chronic EtOH exposure in other cell types and preparations. Acute ethanol exposure inhibits IP_3-mediated Ca^{2+} mobilization in hippocampal pyramidal neurons (S. L. Mironov and A. Hermann, 1996), hepatocytes (D. C. Renard-Rooney et al., 1997), neuroblastoma cells in culture (C. Larsson et al., 1998) and cerebellar microsomes (M. Mezna et al., 1996), generally by decreasing either IP_3 receptor sensitivity or the size of the IP_3 releasable Ca^{2+} pool. On the other hand, withdrawal from chronic EtOH exposure enhances IP_3-mediated Ca^{2+} signaling in Purkinje neurons (J. G. Netzeband et al., 2002), epithelial cells (S. Bokkala et al., 1999) and hepatocytes (T. Nomura et al., 1996; K. Saso et al., 1997). Given the importance of Ca^{2+} signaling mechanisms in synaptic plasticity in DA neurons, these findings suggest that IP_3-mediated Ca^{2+} release may represent a novel target through which ethanol exposure exerts a profound effect on DA neuron plasticity and reward learning.

1.6 HYPOTHESIS AND SPECIFIC AIMS

Development of addiction is thought to involve a maladaptive form of learning and memory in which drug-related experiences are remembered powerfully, resulting in persistent and uncontrollable drug seeking behavior (S. E. Hyman et al., 2006; J. A. Kauer and R. C. Malenka, 2007). Paradoxically, however, the deleterious effects of

alcohol on learning and memory processes are well established. These effects extend to the synaptic correlates of learning and memory, LTP and LTD, which are also inhibited by ethanol throughout the brain. Thus, it currently remains unclear how ethanol exposure can promote the formation of persistent drug-related associations during the development of addiction.

The mesolimbic dopaminergic system that originates in the ventral tegmental area (VTA) is critically involved in the learning of information related to rewards, including drugs of abuse (W. Schultz, 1998; S. E. Hyman et al., 2006). Both natural rewards and drug rewards, such as ethanol, enhance release of DA in the NAc and other limbic structures where DA release is thought to drive reward learning by modulating synaptic plasticity. In addition to plasticity in target structures, plasticity of glutamatergic transmission onto dopamine neurons within the VTA may also play an important role in the development of drug addiction, as these inputs exert a powerful regulatory influence on DA neuron firing patterns (J. A. Kauer and R. C. Malenka, 2007).

Plasticity of NMDA receptor-mediated transmission may be of particular interest, as NMDAR activation in the VTA is necessary for DA neuron burst firing and phasic DA release in projection areas that occurs in response to rewards or reward-predicting stimuli (L. A. Sombers et al., 2009; L. S. Zweifel et al., 2009). A recent study identified a novel form of LTP of NMDAR EPSCs that is induced by sustained glutamatergic input paired with postsynaptic burst firing (M. T. Harnett et al., 2009). Induction of this LTP is dependent upon amplification of AP-evoked Ca^{2+} signals by preceding activation of mGluRs. This amplification is dependent on Ca^{2+} release from intracellular stores, where

IP_3 generated by mGluR activation increases Ca^{2+}-induced Ca^{2+} release triggered by AP-induced Ca^{2+} influx (G. Cui et al., 2007).

Long-term ethanol treatment has been shown to produce an enhancement of IP_3-mediated Ca^{2+} signaling in different cell types (T. Nomura et al., 1996; K. Saso et al., 1997; J. G. Netzeband et al., 2002). Therefore, the overriding hypothesis of this dissertation is that repeated *in vivo* ethanol exposure enhances IP_3-mediated Ca^{2+} signaling in VTA DA neurons. Such an enhancement should act as a form of metaplasticity, resulting in DA neurons that are more susceptible to induction of LTP of NMDA receptor-mediated transmission.

The first specific aim will test the hypothesis that repeated *in vivo* ethanol exposure enhances mGluR-mediated facilitation of AP-associated Ca^{2+} signals in DA neurons. The ability of IP_3 to amplify AP-associated Ca^{2+} signals will be compared between saline and ethanol treated animals. The magnitude of LTP of NMDA receptor-mediated transmission will also be assessed and compared between groups. Finally, the effect of previous ethanol exposure on subsequent reward learning will be tested using the CPP paradigm.

The second specific aim will test the hypothesis that exposure to psychostimulant drugs also enhances IP_3-mediated Ca^{2+} release from intracellular stores. Such a finding would suggest that alterations in intracellular Ca^{2+} signaling may be a common neuroadaptation in response to other drugs that elevate DA concentrations in the VTA.

Chapter 2: Materials and Methods

2.1 ANIMALS

Male C57BL/6J mice (3-4 weeks old) were obtained from Jackson Laboratory. Male Sprague Dawley rats (4-6 weeks old) were obtained from Harlan. All animals were housed under a 12-h light/dark cycle (lights on at 7:00 AM). Food and water were available *ad libitum*. All animal procedures were approved by the Universtiy of Texas Institutional Animal Care and Use Committee.

2.2 *IN VIVO* DRUG TREATMENT

Mice received i.p. injections of ethanol (2 g/kg, 20% vol/vol) or an equivalent volume of saline, 3 times per day (3-3.5 hrs apart) for 7 days. Mice were returned to the home cage immediately after each injection. Rats received once-daily i.p. injections of $_{D}$-amphetamine sulfate (5 mg/kg) or an equivalent volume of saline for 7 days. Injections were performed in a chamber different from the home cage where the rats were kept for 20 min before being placed back in the home cage.

2.3 SLICES AND SOLUTIONS

Horizontal midbrain slices (190-210 μm) containing the VTA were prepared 1, 7, 10, or 28-35 days following the last saline/ethanol/amphetamine injection. Slices were cut in an ice-cold solution containing (in mM) 205 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 7.5 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose, and 25 $NaHCO_3$, saturated with 95% O_2 and 5% CO_2 (~305 mOsm/kg) and incubated > 1hr at 35° C in a solution containing (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$, saturated with 95% O_2 and 5% CO_2 (pH 7.4, ~295 mOsm/kg). Recordings were made at 34-35°C in the same solution perfused at ~2.5 ml/min. The pipette solution contained (in mM) 115 K-methylsulfate or K-gluconate, 20 KCl, 1.5 $MgCl_2$, 10 HEPES, 0.025 EGTA, 2 Mg-ATP, 0.2 Na_2-GTP, and 10 Na_2-phosphocreatine (pH 7.25, ~280 mOsm/kg).

2.4 ELECTROPHYSIOLOGICAL RECORDINGS

Cells were visualized using an upright microscope (Olympus) with infrared/differential interference contrast optics. Recordings were made in the lateral VTA located between 50-150 μm from the medial border of the medial terminal nucleus of the accessory optic tract. Putative dopamine neurons were identified by their spontaneous firing (1-5 Hz) with broad APs (>1.2 ms) recorded in cell-attached configuration and large I_h currents (> 200 pA elicited by 1.5-s hyperpolarizing steps from -62 mV to -112 mV) in whole-cell configuration. Whole-cell voltage-clamp recordings

were made at a holding potential of -62 mV, corrected for a liquid junction potential of -7 mV. Pipettes with tip resistance of 1.5-2.0 MΩ were used. Series and input resistances were monitored throughout experiments and recordings were discarded if the series resistance increased beyond 20 MΩ or the input resistance dropped below 200 MΩ. A Multiclamp 700A amplifier (Molecular Devices) and Axograph X (AxoGraph Scientific) were used to record and collect data, which were filtered at 1-5 kHz and digitized at 2-10 kHz.

Unclamped APs were evoked with 2-ms depolarizing pulses from -62 mV to -7 mV. The time integral of the SK-mediated tail outward current (I_{KCa}), measured between 20 ms and 400-1000 ms following the depolarizing pulse, was used to assess AP-evoked Ca^{2+} rises, as has been done previously (G. Cui et al., 2007; K. C. Ahn et al., 2010). For experiments with amphetamine and saline-treated rats I_{KCa} integrals were normalized by membrane capacitance and reported as picocoulomb/picofarad.

2.5 FLASH PHOTOLYSIS

Caged IP_3 (25-200 μM; Invitrogen) was loaded into the cytosol through the whole-cell pipette. A 1-ms UV pulse was applied using a xenon arc lamp (Cairn Research) to rapidly release IP_3. The UV pulse was focused through a 60X objective onto a ~350-μm diameter area centered at the recorded neurons. The amount of photolysis is known to be proportional to the UV pulse intensity (J. A. McCray et al.,

1980), which is proportional to the capacitance of the capacitor feeding current to the flash lamp. UV pulse intensity is therefore reported in units of capacitance (50-4050 μF).

2.6 LTP EXPERIMENTS

Synaptic stimuli were applied every 20 s using bipolar tungsten electrodes (~100-μm tip separation) placed 50-150 μm rostral to the recorded neuron. To isolate NMDAR EPSCs, recordings were performed in the presence of DNQX (10 μM), picrotoxin (100 μM), and eticlopride (100 nM) to block AMPA, $GABA_A$, and D_2 dopamine receptors. LTP experiments in EtOH and saline-treated mice were performed in physiological Mg^{2+} (1.2 mM). NMDAR EPSCs evoked in this manner are entirely blocked by the NMDA antagonist AP-5 (M. T. Harnett et al., 2009).

For LTP induction, sustained synaptic stimulation (70 stimuli at 50 Hz) was paired with a burst of 5 APs at 20 Hz, with the burst onset delayed 1 s from the onset of the synaptic stimulation train. This synaptic stimulation-burst pairing was repeated 10 times every 20 s. Magnitude of LTP was determined by comparing the average EPSC amplitude over the 10-min baseline period with that during another 10-min window ~30-40 min after LTP induction.

2.7 CONDITIONED PLACE PREFERENCE

A CPP box consisting of two distinct compartments, separated by a small middle chamber, was used for conditioning (Med Associates). One compartment had a mesh floor with white walls, while the other had a grid floor with black walls. One day after the 7-day saline/ethanol treatment in the home cage, mice were subjected to a pretest in a conditioning room, in which they were allowed to freely explore the entire CPP box for 20 min. The percentage of time spent in each compartment was determined after excluding the time spent in the middle chamber. Any mice that spent more than 60% of time in either compartment during the pretest were not used for conditioning. For cocaine CPP, mice were subjected to 2-day conditioning starting the next day, in which one of the two compartments was randomly assigned as the cocaine-paired side in each mouse. Here, mice were given an injection of cocaine (5 mg/kg, i.p.) and confined to one compartment for 30 min in one trial and given a saline injection and confined to the other compartment for 30 min in another trial. The two trials were separated by at least 4 hrs. The order of cocaine/saline trials was reversed on the second day of conditioning. A 20-min posttest was performed the following day. Mice that spent more than 8 min in the middle chamber during pretest or posttest were excluded from analysis. CPP score was determined by subtracting the preference for the cocaine-paired side in the pretest from that in the posttest. For ethanol CPP, mice were subjected to 4-day conditioning in which they were given either ethanol (2 g/kg, i.p.) or saline and confined to one compartment for 5 min.

2.8 DRUGS

DHPG, CRF, DNQX, eticlopride and K41498 were obtained from Tocris Bioscience. Caged IP_3 was purchased from Invitrogen. All other chemicals were from Sigma-RBI.

2.9 DATA ANALYSIS

Data are expressed as mean ± SEM. Statistical significance was determined by Student's *t* test or ANOVA followed by Bonferroni *post hoc* test. The difference was considered significant at $P < 0.05$.

Chapter 3: Results

3.1 AIM 1: REPEATED *IN VIVO* ETHANOL EXPOSURE ENHANCES SYNAPTIC PLASTICITY IN VENTRAL TEGMENTAL AREA DOPAMINE NEURONS

3.1.1 Repeated ethanol exposure enhances mGluR-mediated facilitation of AP-associated Ca^{2+} signals

Activation of phosphoinositide (PI)-coupled receptors, such as mGluRs, facilitates AP-evoked Ca^{2+} signals in DA neurons via IP_3 receptor-mediated release of Ca^{2+} from intracellular stores (G. Cui et al., 2007). This mGluR/IP_3-induced facilitation of AP-evoked Ca^{2+} signals is required for the induction of NMDAR LTP in dopamine neurons (M. T. Harnett et al., 2009). Thus, we first tested if *in vivo* ethanol exposure alters the effect of the mGluR agonist DHPG on AP-evoked Ca^{2+} signals, which were assessed in whole-cell voltage clamp recordings by measuring the small-conductance Ca^{2+}-sensitive K^+ (SK) current ($I_{K(Ca)}$) following unclamped APs evoked by brief depolarizing pulses (See Methods). While bath perfusion of DHPG (3 μM) produced small facilitation of $I_{K(Ca)}$ in VTA dopamine neurons from naïve or saline-treated mice (Fig. 5A and B), this DHPG effect was significantly increased 1 day after withdrawal from 7-day ethanol treatment (2 g/kg, i.p., 3 times daily). This enhancement of the DHPG effect persisted following 7 days of withdrawal from ethanol treatment (Fig. 5C). However, following 4-5 weeks of withdrawal the magnitude of DHPG-induced facilitation of $I_{K(Ca)}$ returned to a level equivalent to naïve age-matched controls (8-9 weeks old) (Fig. 5C). There was no

change in basal $I_{K(Ca)}$ amplitude measured prior to DHPG perfusion (Fig. 5D), suggesting SK channels themselves are not affected by ethanol treatment. Furthermore, DHPG-induced inward currents, which are independent of IP_3 (G. Cui et al., 2007), were not altered by ethanol exposure (Fig. 5E), suggesting that the increase in DHPG effect on $I_{K(Ca)}$ results from changes in IP_3 signaling downstream of mGluRs.

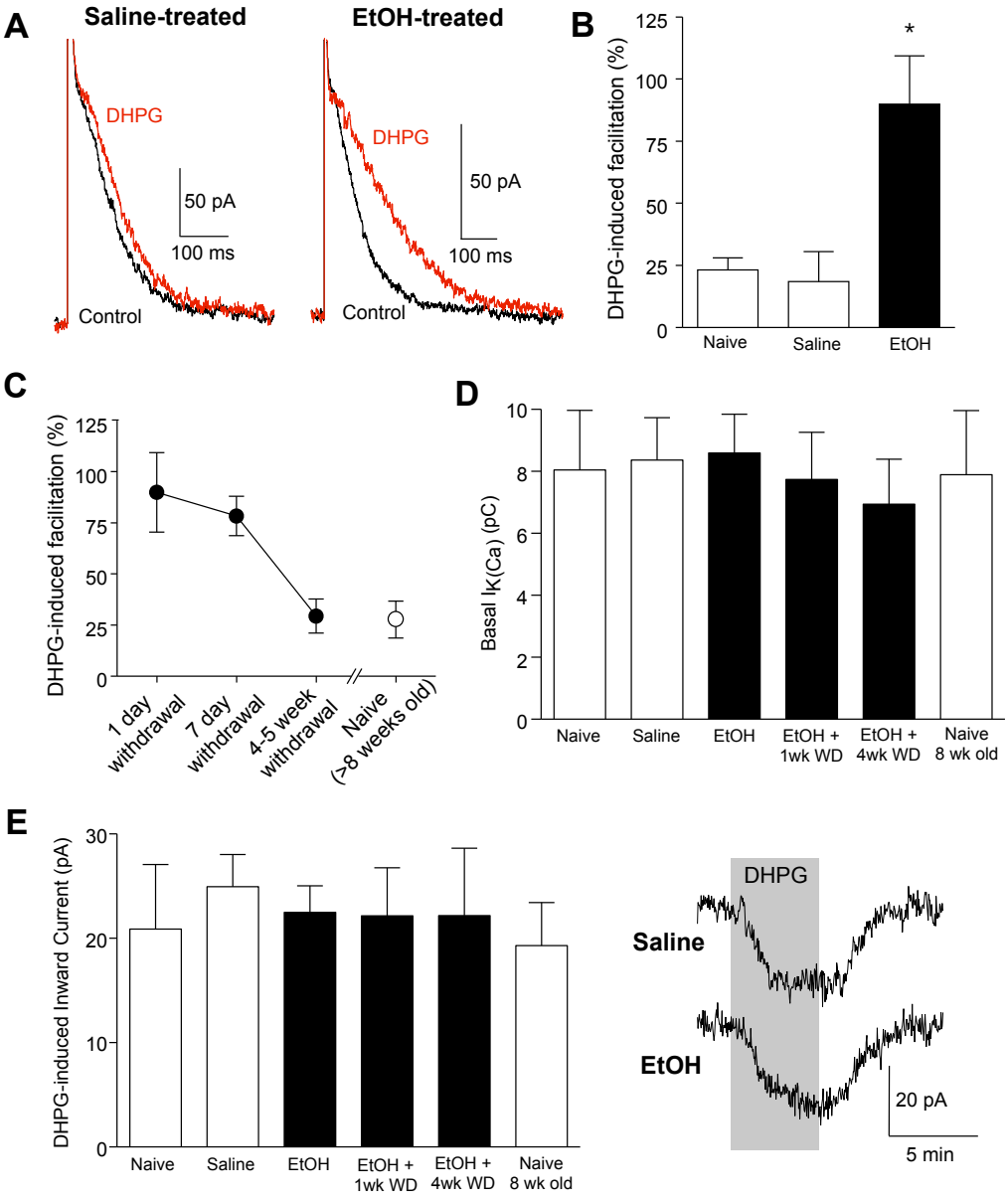

A
Saline-treated
EtOH-treated
DHPG
50 pA
100 ms
Control
B
DHPG-induced facilitation (%)
125
100
75
50
25
0
*
Naive
Saline
EtOH
C
DHPG-induced facilitation (%)
1 day withdrawal
7 day withdrawal
4-5 week withdrawal
Naive (>8 weeks old)
D
Basal IK(Ca) (pC)
Naive
Saline
EtOH
EtOH + 1wk WD
EtOH + 4wk WD
Naive 8 wk old
E
DHPG-induced Inward Current (pA)
Naive
Saline
EtOH
EtOH + 1wk WD
EtOH + 4wk WD
Naive 8 wk old
DHPG
Saline
EtOH
20 pA
5 min

Figure 5. Repeated ethanol exposure enhances mGluR-mediated facilitation of AP-associated Ca^{2+} signals.

(A) Example traces of $I_{K(Ca)}$ illustrating the effects of DHPG (3 μM) on $I_{K(Ca)}$ in cells from saline and ethanol-treated mice (1 day withdrawal). (B) Summary bar graph demonstrating that *in vivo* ethanol exposure augmented DHPG-induced facilitation of $I_{K(Ca)}$ (naïve group: 7 cells from 4 mice, saline group: 10 cells from 5 mice, ethanol group: 14 cells from 8 mice; $F_{2,28} = 6.25$, $p < 0.01$, one-way ANOVA). $^{*}p < 0.05$ versus naïve and saline groups. (C) Summary graph depicting DHPG effect on $I_{K(Ca)}$ after different periods of ethanol withdrawal (1 day withdrawal group: 14 cells from 8 mice, 7 day withdrawal group: 7 cells from 5 mice, 4-5 week withdrawal group: 10 cells from 5 mice, naïve (>8 weeks old) group: 9 cells from 5 mice). (D) The size of basal $I_{K(Ca)}$ was not altered after ethanol treatment regardless of length of withdrawal. (E) DHPG-induced inward currents were not affected by ethanol treatment. Right, example traces of DHPG-induced currents in cells from saline- and ethanol-treated mice. DHPG was perfused at the time indicated. The data in (D) and (E) were obtained from the same cells shown in (A) and (B).

3.1.2 Repeated ethanol exposure enhances IP_3 receptor sensitivity

To more directly examine the alterations in IP_3-mediated release of Ca^{2+} from intracellular stores induced by ethanol exposure, we next performed flash photolysis of caged IP_3 (200 μM) and measured the resulting SK-mediated outward currents (I_{IP3}) (H. Morikawa et al., 2000). The concentration of IP_3 released within the recorded neuron was varied by applying different UV flash intensities. This is achieved by adjusting the capacitance (up to 4050 μF) of the capacitor in the power supply of the photolysis system. A series of IP_3-mediated currents were recorded and the IP_3 concentration-response curve constructed in each cell was fitted to a logistic equation (Fig. 6A and B). The EC_{50} value [i.e., the UV intensity (expressed in μF) producing half-maximal current] and the maximal I_{IP3} amplitude were obtained. The average EC_{50} value was significantly lower in ethanol-treated mice compared to controls (Fig. 6C), while there was no difference in the maximal I_{IP3} amplitude (Fig. 6D). These results demonstrate that repeated ethanol exposure increases IP_3 sensitivity in VTA dopamine neurons.

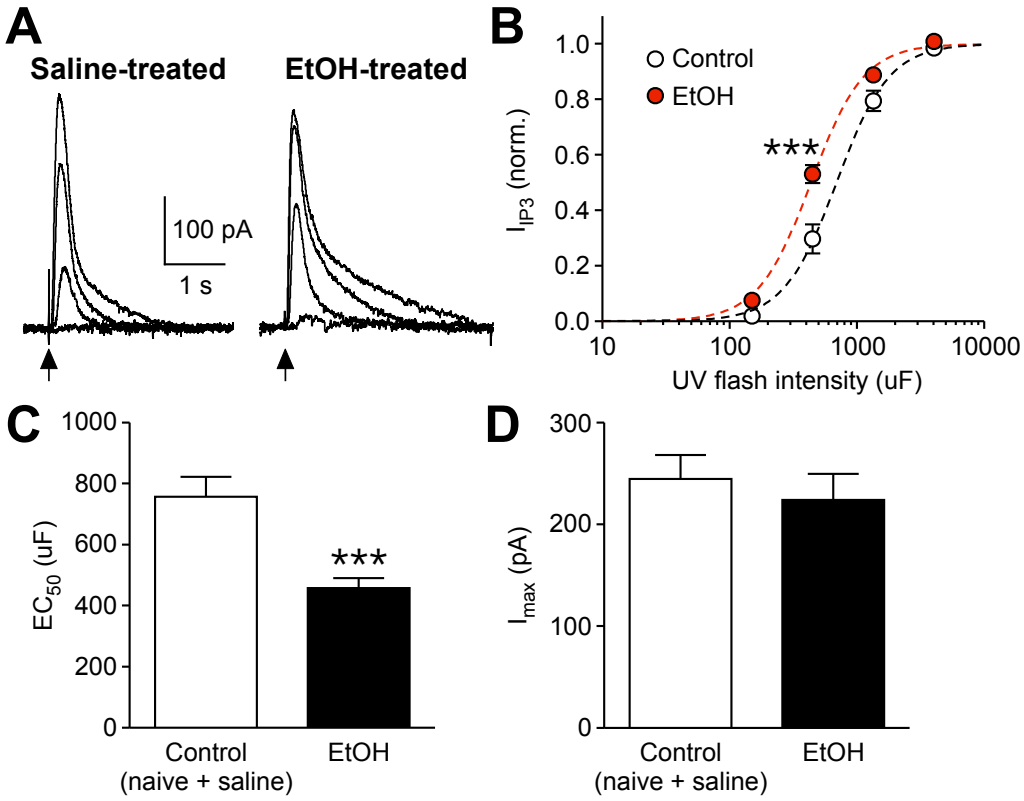


Figure 6. Repeated ethanol exposure increases IP_3 sensitivity.

(A) Traces of I_{IP3} evoked with different UV pulse intensities (150, 450, 1350, and 4050 μF) in VTA neurons from saline- and ethanol-treated mice. These cells were loaded with caged IP_3 (200 μM). UV flashes were applied at the time indicated by the arrow. (B) Averaged concentration (UV flash intensity)-response (I_{IP3}) curves from control and ethanol-treated mice. The I_{IP3} amplitude was normalized to the maximal value (estimated from fit to a logistic equation) in each cell. Data from naïve and saline-treated mice were pooled as a control group (control group: 13 cells from 7 naïve mice and 6 cells from 4 saline-treated mice, ethanol group: 16 cells from 10 mice; group: $F_{1,99} = 12.7$, $p < 0.01$; flash intensity: $F_{3,99} = 643.1$, $p < 0.0001$; group · flash intensity: $F_{3,99} = 7.45$, $p < 0.001$, mixed two-way ANOVA). Dashed lines are fit to a logistic equation. ***$p < 0.001$ versus control. (C) Summary bar graph showing that EC_{50} values were reduced in ethanol-treated mice ($t_{33} = 3.88$, $p < 0.001$, unpaired t test). (D) The maximal I_{IP3} amplitude was not altered by *in vivo* ethanol treatment.

3.1.3 The cAMP/PKA pathway modulates IP_3 receptor sensitivity in DA neurons

PKA-mediated phosphorylation of IP_3Rs is known to increase their IP_3 sensitivity (L. E. Wagner, 2nd et al., 2008). To determine whether phosphorylation of IP_3 receptors by PKA alters IP_3 receptor sensitivity in DA neurons, we tested the effect of forskolin, a potent activator of adenylyl cyclase, on I_{IP3} in naïve mice. Bath perfusion of forskolin (3 μM) caused a significant increase in I_{IP3} evoked with an EC_{50} flash intensity, while there was minimal enhancement of I_{IP3} evoked with a maximal flash intensity (Fig. 7A and B). Therefore, activation of the cAMP-PKA pathway increases IP_3R sensitivity in a manner analogous to repeated ethanol exposure. We further tested forskolin (3-10 μM) on AP-evoked $I_{K(Ca)}$ and found that it had no effect (Fig. 7C and D), similarly to the lack of effect of *in vivo* ethanol treatment on $I_{K(Ca)}$ (Fig. 5D).

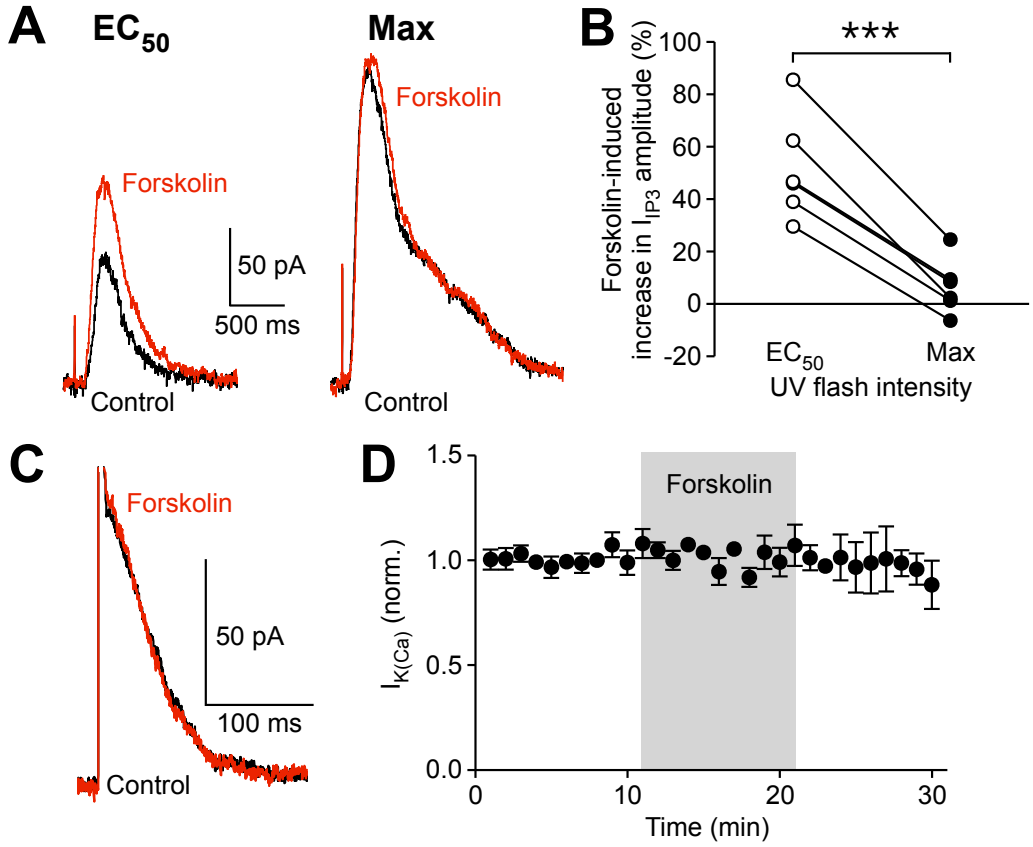


Figure 7. Stimulation of the cAMP-PKA pathway increases IP_3 sensitivity.

(A) Representative traces of I_{IP3} in control (black) and forskolin (3 µM; red). Traces on the left were elicited with an EC_{50} flash intensity, while those on the right represent maximal I_{IP3} evoked in the same cell. The cell was loaded with caged IP_3 (200 µM). (B) Effects of forskolin on I_{IP3} evoked with EC_{50} or maximal flash intensities are plotted in 6 cells ($t_5 = 9.07$, $p < 0.001$, paired t test). (C) Example traces of $I_{K(Ca)}$ in control (black) and forskolin (10 µM; red). (D) Summary time graph demonstrating that forskolin (3-10 µM) failed to affect $I_{K(Ca)}$ ($n = 5$).

In order to further examine the role of PKA phosphorylation of IP_3 receptors we filled neurons with a PKA inhibitory peptide PKI (100-200 μM) through the patch pipette. We first assessed the effect of intracellular PKI on I_{IP3} evoked by flash photolysis of caged IP_3 (100 μM). As described earlier, a series of IP_3-mediated currents were recorded and the IP_3 concentration-response curve constructed in each cell was fitted to a logistic equation (Fig. 8A and B). The average EC_{50} value was significantly higher in neurons loaded with PKI (Fig. 8C). These results suggest that a tonic level of PKA activation increases IP_3 sensitivity in VTA dopamine neurons.

Additionally, we found a significant decrease in the magnitude of DHPG-induced facilitation of $I_{K(Ca)}$ in dopamine neurons loaded with PKI as compared to neurons filled with control internal solution (Fig. 8D and E). In PKI-loaded neurons, higher concentrations of DHPG (5-10 μM) were able to significantly increase facilitation of $I_{K(Ca)}$ (Fig. 8D and F), consistent with the idea that PKA modulates IP_3 sensitivity in dopamine neurons.

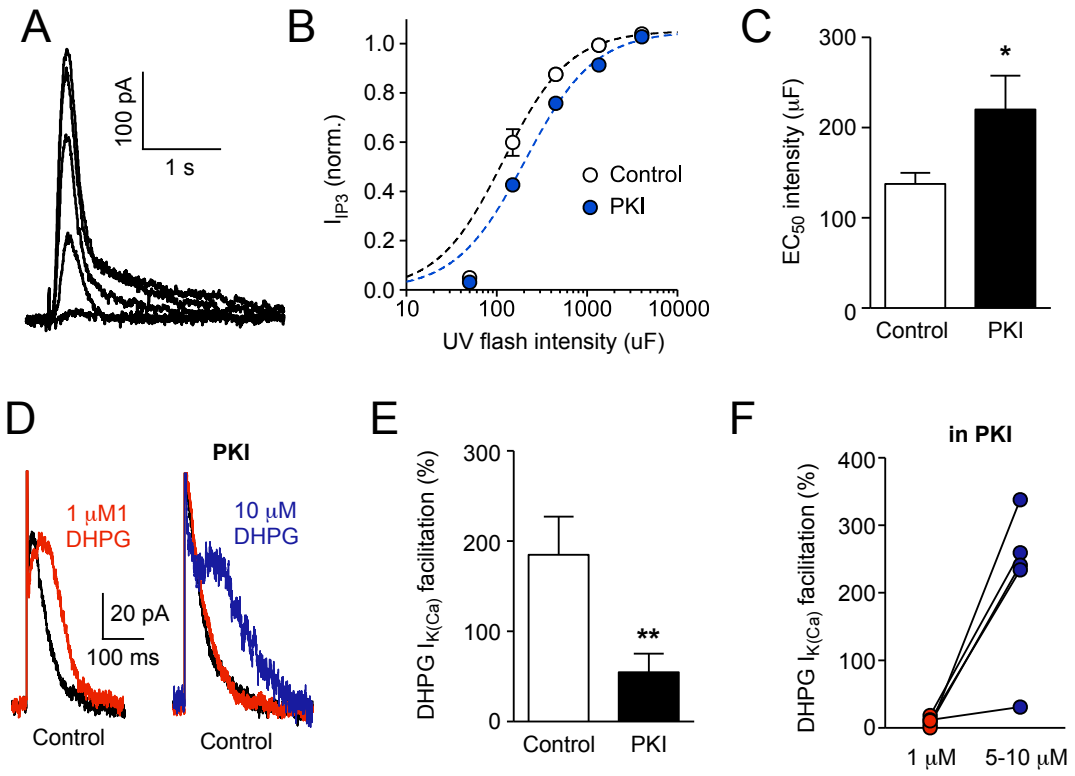


Figure 8. PKA modulates IP_3 receptor sensitivity in DA neurons.

(A) Sample traces of I_{IP3} evoked with different UV pulse intensities (50, 150, 450, 1350, and 4050 μF) in neurons loaded with PKI (200 μM) and caged IP_3 (200 μM). UV flashes were applied at the time indicated by the arrow. (B) Averaged concentration (UV flash intensity)-response (I_{IP3}) curves from control and PKI-loaded neurons. The I_{IP3} amplitude was normalized to the maximal value (estimated from fit to a logistic equation) in each cell. (C) Summary bar graph showing that EC_{50} values were incresed in PKI loaded neurons (control: n = 5, PKI: n = 7, $p < 0.05$, unpaired *t* test). (D) Sample traces demonstrating the effect of DHPG on $I_{K(Ca)}$ in either control internal solution (left) or PKI (right). A higher concentration of DHPG (10 μM) restored facilitation of $I_{K(Ca)}$ in this PKI-loaded neuron (blue trace). (E) Summary bar graph showing that the effect of DHPG on $I_{K(Ca)}$ is reduced in PKI (control; n = 13, PKI: n = 10, $p < 0.01$, unpaired t-test). (F) The effects of 1 μM vs. 5-10 μM DHPG are shown in 5 PKI-filled neurons (n = 5, $p < 0.05$, paired t-test).

3.1.4 Repeated ethanol exposure enhances IP_3-mediated Ca^{2+} signaling via PKA

It has been shown previously that *in vivo* ethanol exposure causes upregulation of the adenylyl cyclase-cAMP-PKA pathway in the mesolimbic dopamine system (J. Ortiz et al., 1995; M. Melis et al., 2002). To determine whether increased phosphorylation of IP_3 receptors by PKA underlies the enhanced IP_3 receptor sensitivity and mGluR-mediated facilitation of $I_{K(Ca)}$ seen in the EtOH treated animals, we tested the effect of a PKA inhibitor H89 (10 μM) on the ability of IP_3 to facilitate single AP-evoked $I_{K(Ca)}$ in treated animals. In these experiments, dopamine neurons were filled with a low concentration of caged IP_3 (25 μM) and subjected to a subthreshold UV flash (100 μF) that produced no measurable outward current by itself. This subthreshold flash caused significant facilitation of $I_{K(Ca)}$ when applied 50 ms prior to an AP (Fig. 9A-C). The flash intensity was held constant in order to compare the magnitude of $I_{K(Ca)}$ facilitation caused by the same concentration of IP_3 in different cells. Consistent with the results from DHPG experiments (Fig. 5A and B), IP_3-induced facilitation of $I_{K(Ca)}$ was significantly larger in ethanol-treated mice compared to saline-treated controls (Fig. 9A-C). Treatment with the PKA inhibitor H89 (10 μM, >1 hr preincubation plus intracellular application through patch pipette) reversed the increase in $I_{K(Ca)}$ facilitation following *in vivo* ethanol exposure (Fig. 9B and C). Notably, H89 treatment had no effect on basal $I_{K(Ca)}$ not facilitated by IP_3 (Fig. 9D).

3.1.5 CRF further amplifies IP_3-mediated facilitation of AP-evoked $I_{K(Ca)}$

Increased activity of the CRF system is believed to be an important neuroadaptation to exposure to drugs of abuse (M. Heilig and G. F. Koob, 2007). Indeed, repeated ethanol exposure increases CRF levels in the brain (Z. Sarnyai et al., 2001; M. Heilig and G. F. Koob, 2007). Furthermore, CRF has been shown to potentiate mGluR-induced intracellular Ca^{2+} release via a PKA-dependent mechanism in dopamine neurons (A. C. Riegel and J. T. Williams, 2008). To examine the effects of CRF on IP_3 signaling in saline- and ethanol-treated mice we perfused CRF (300 nM) during the recordings described above, in which weak UV flashes to uncage IP_3 were paired with APs and the ability of subthreshold concentrations of IP_3 to facilitate $I_{K(Ca)}$ was measured. In saline-treated animals, bath perfusion of CRF (300 nM) significantly increased the magnitude of IP_3-induced facilitation of $I_{K(Ca)}$ (Fig. 9A and C). Furthermore, CRF was able to cause a robust additional increase in $I_{K(Ca)}$ that was already greatly facilitated by IP_3 in ethanol-treated mice (Fig. 9B and C). Treatment with the PKA inhibitor H89 (10 μM, >1 hr preincubation plus intracellular application through patch pipette) completely abolished the effect of CRF (Fig. 9B and C). Notably, CRF application had no effect on basal $I_{K(Ca)}$ not facilitated by IP_3 (Fig. 9D, also note the overlap of gray and orange traces in Fig. 9A and B). Additionally, we found that the selective $CRFR_2$ antagonist K41498 (100-300 nM) blocked the enhancement of IP_3-induced facilitation of $I_{K(Ca)}$ by CRF in ethanol treated mice (Fig. 9E). K41498 itself had no effect on the magnitude of IP_3-induced

facilitation of $I_{K(Ca)}$, indicating that the effect of ethanol treatment is not mediated by an increased CRF tone in the slice (Fig. 9E). These data demonstrate that previous *in vivo* ethanol exposure and acute CRF application converge on PKA to amplify the facilitatory effect of IP_3 on AP-evoked Ca^{2+} signals.

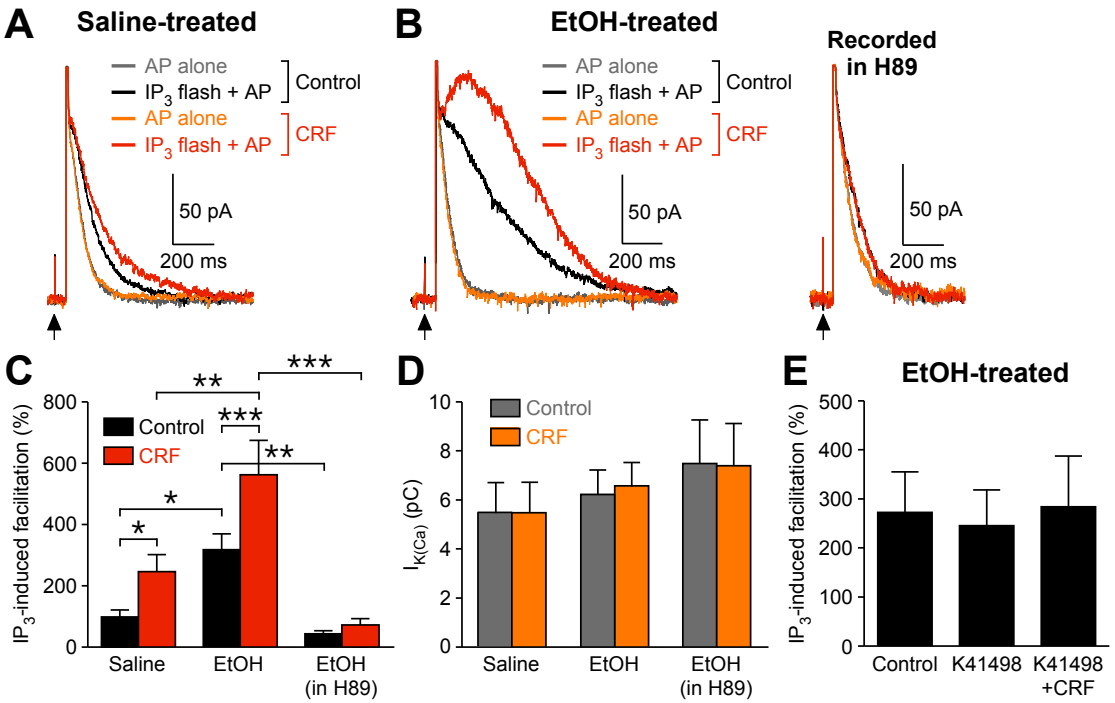


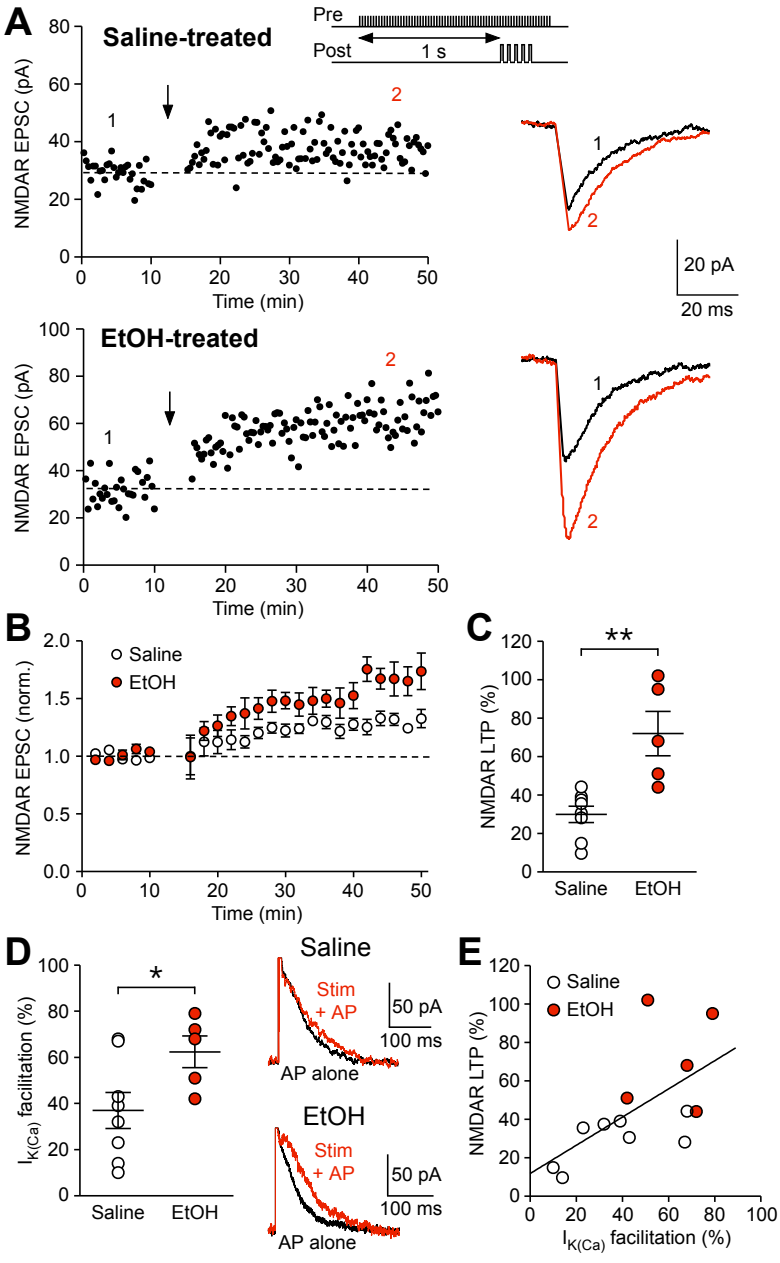
Figure 9. CRF amplifies the increase in IP_3 effect on AP-evoked Ca^{2+} signals produced by *in vivo* ethanol exposure.

(A) Representative traces of $I_{K(Ca)}$, evoked by itself (gray and orange traces) or paired with preceding photolysis of caged IP_3 (25 µM; black and red traces), in a saline-treated mouse. A low-intensity UV flash (100 µF) was applied at the arrow (50 ms before the AP). The magnitude of IP_3-induced facilitation of $I_{K(Ca)}$ is determined by comparing gray and black traces in control solution and by comparing orange and red traces in CRF (300 nM). Note that bath perfusion of CRF, which failed to affect $I_{K(Ca)}$ itself, was capable of augmenting IP_3-induced facilitation. (B) Representative traces of $I_{K(Ca)}$, evoked as in (A), from an ethanol-treated mouse. IP_3 produced robust facilitation of $I_{K(Ca)}$ (black trace), which was further augmented by CRF (red trace). Treatment with H89 (10 µM) largely suppressed IP_3-induced $I_{K(Ca)}$ facilitation and abolished the CRF effect. (C) Summary bar graph plotting the magnitude of IP_3-induced facilitation of $I_{K(Ca)}$ under the conditions illustrated in (A) and (B) (saline group: 8 cells from 4 mice, ethanol group: 6 cells from 4 mice, ethanol group recorded in H89: 6 cells from 5 mice; group: $F_{2,17} = 11.4$, $P < 0.001$; CRF: $F_{1,17} = 21.1$, $p < 0.001$; group × CRF: $F_{2,17} = 4.38$, $p < 0.05$, mixed two-way ANOVA). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (D) Summary bar graph depicting the size of $I_{K(Ca)}$ (without IP_3). These data were from the same cells shown in (C). (E) The CRF_2 receptor antagonist K41498 blocked the augmentation of IP_3-induced facilitation of $I_{K(Ca)}$ by CRF (5 cells from 3 ethanol-treated mice).

3.1.6 *In vivo* ethanol exposure promotes NMDAR plasticity in VTA DA neurons

LTP of NMDAR-mediated transmission onto DA neurons is dependent on mGluR-mediated facilitation of burst-evoked Ca^{2+} release from intracellular stores (M. T. Harnett et al., 2009). Therefore, we next examined if repeated ethanol exposure affects NMDAR plasticity in VTA dopamine neurons. Pharmacologically isolated NMDAR EPSCs were recorded at -62 mV in physiological Mg^{2+} (1.2 mM) solution (see Methods). Previous studies using C57BL/6J mice have found no global changes in NMDAR function in VTA dopamine neurons after repeated *in vivo* injections of ethanol (M. S. Brodie, 2002; F. W. Hopf et al., 2007). Therefore, we normalized synaptic stimulation intensity using the NMDAR EPSC amplitude (~20-30 pA; saline group: 26 ± 2 pA, 8 cells from 7 mice; ethanol group: 27 ± 3 pA, 5 cells from 5 mice). Following 10-min baseline recording, LTP was induced by repetitively pairing (10 times every 20 s) sustained synaptic stimulation (70 stimuli at 50 Hz) with a burst of 5 APs at 20 Hz in the postsynaptic neuron. While this pairing protocol resulted in relatively small LTP of NMDAR EPSCs in saline-treated controls (30 ± 4%), ethanol-treated animals exhibited significantly larger magnitude of LTP (72 ± 11%; $t_{11} = 4.04$, $P < 0.01$ vs. saline group, unpaired *t* test) (Fig. 10A-C). Prior to running the LTP induction protocol, facilitation of $I_{K(Ca)}$ by synaptic stimulation was assessed in each neuron by evoking an AP 60 ms after the end of a 1 s stimulation train. We found that facilitation of $I_{K(Ca)}$ by synaptic stimulation was significantly increased in neurons from ethanol-treated animals (Fig. 10D) (saline group: 37 ± 8%, ethanol group: 62 ± 7%; $t_{11} = 2.25$, $P < 0.05$, unpaired *t*

test). Furthermore, the magnitude of NMDAR LTP was positively correlated with that of $I_{K(Ca)}$ facilitation across neurons from both groups of mice ($r = 0.61$, $P < 0.05$) (Fig. 10E). These data demonstrate that repeated ethanol exposure enhances the induction of NMDAR plasticity, most likely via an increase in mGluR-mediated facilitation of AP-evoked Ca^{2+} signals.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B
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C
NMDAR LTP (%)
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D
*
$I_{K(Ca)}$ facilitation (%)
Stim
+ AP
AP alone
50 pA
100 ms
E
NMDAR LTP (%)
$I_{K(Ca)}$ facilitation (%)

Figure 10. NMDAR-mediated transmission becomes more susceptible to LTP induction after repeated ethanol exposure.

(A) Example experiments to induce NMDAR LTP in saline- and ethanol-treated mice. Time graphs of NMDAR EPSC amplitude are shown on the left. The LTP induction protocol, which consisted of synaptic stimulation-burst pairing (top inset), was delivered at the time indicated by the arrow. Traces of NMDAR EPSCs at times indicated by numbers in the time graphs are shown on the right. (B) Summary time graph of NMDAR LTP experiments (saline group: 8 cells from 7 mice, ethanol group: 5 cells from 5 mice). (C) Summary graph plotting the magnitude of NMDAR LTP in saline- and ethanol-treated mice ($t_{11} = 4.04$, $p < 0.01$, unpaired t test). (D) Summary graph showing that the magnitude of $I_{K(Ca)}$ facilitation produced by preceding synaptic stimulation was larger in ethanol-treated mice ($t_{11} = 2.25$, $p < 0.05$, unpaired t test). Example traces illustrating synaptic facilitation of $I_{K(Ca)}$ are shown on the right. (E) The magnitude of NMDAR LTP is plotted versus the magnitude of synaptic facilitation of $I_{K(Ca)}$ in the cells shown in . Solid line is a linear fit to all data points from both saline- and ethanol-treated mice. The data summarized in (B)-(E) were all from the same cells.

3.1.7 *In vivo* ethanol exposure enhances subsequent cocaine-conditioned place preference

Enhanced NMDAR plasticity in VTA dopamine neurons may facilitate the learning of environmental stimuli associated with rewards, including drugs of abuse (K. C. Ahn et al., 2010). Thus, we tested the effect of repeated ethanol exposure on subsequent reward learning using cocaine-induced CPP, a form of behavioral conditioning that is dependent on NMDARs in the VTA (G. C. Harris et al., 2004; L. S. Zweifel et al., 2008). Mice were first treated with either saline or ethanol (2 g/kg, i.p.) for 7 days, as described above. Then, after initial preference for the two compartments of the CPP box was determined on the pretest day, mice were subjected to 2-day conditioning in which cocaine (5 mg/kg, i.p.) was paired with one compartment while saline was paired with the other compartment on each day. During the CPP posttest, ethanol-treated mice exhibited a significantly greater increase in preference for the cocaine-paired compartment (Fig. 11A and B). These data demonstrate that repeated ethanol exposure enhances subsequent learning of cocaine-associated environmental stimuli.

We further asked if repeated ethanol exposure could affect subsequent ethanol CPP. To test this, mice underwent 4-day CPP conditioning with ethanol (2 g/kg, i.p.) after 7-day saline/ethanol treatment. Neither group developed significant preference for the ethanol-paired compartment (Fig. 11C and D), in agreement with a previous report

showing that C57BL/6J mice do not readily develop ethanol CPP (C. L. Cunningham et al., 1992).

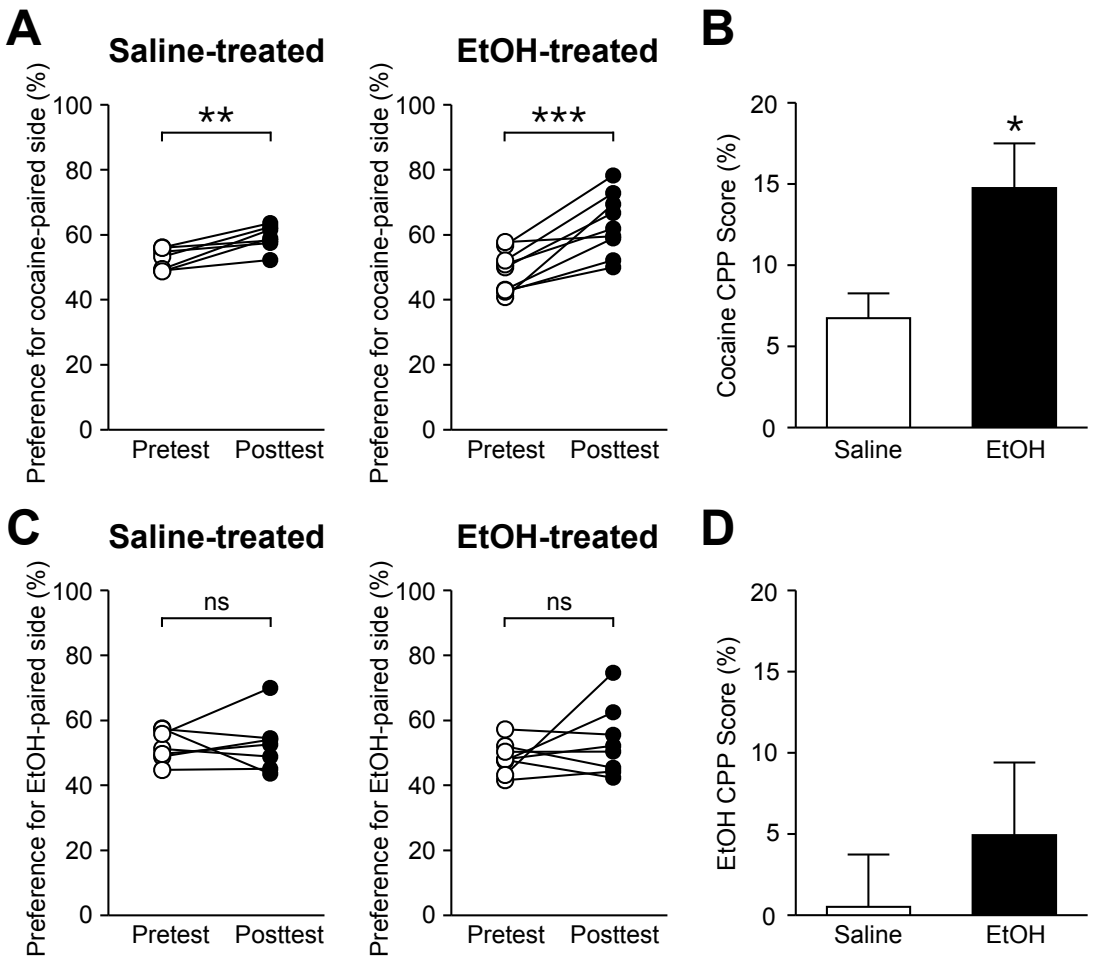

Figure 11. Previous ethanol exposure promotes cocaine-induced CPP.

(A) Changes in the preference for the cocaine-paired side after 2-day conditioning are shown for saline- and ethanol-treated mice (saline group: $t_6 = 4.46$, $p < 0.01$; ethanol group: $t_8 = 5.39$, $p < 0.001$, paired t test). (B) Summary bar graph showing that cocaine CPP is enhanced in ethanol-treated mice ($t_{14} = 2.35$, $p < 0.05$, unpaired t test). (C) Changes in the preference for the ethanol-paired side after 4-day conditioning are shown for saline- and ethanol-treated mice (saline group: $t_6 = 0.16$, $p = 0.88$; ethanol group: $t_7 = 1.10$, $p = 0.31$, paired t test). (D) Summary bar graph of ethanol CPP experiments ($t_{13} = 0.78$, $p = 0.45$, unpaired t test).

3.2 AIM 2: REPEATED *IN VIVO* AMPHETAMINE EXPOSURE ENHANCES IP_3R-MEDIATED CA^{2+} SIGNALING IN VENTRAL TEGMENTAL AREA DOPAMINE NEURONS

3.2.1 *In vivo* amphetamine exposure enhances IP_3 receptor sensitivity

To directly examine the effects of repeated amphetamine exposure on IP_3-mediated Ca^{2+} signaling we performed flash photolysis of caged IP_3 (100 μM) and measured the resulting SK-mediated I_{IP3}. A series of IP_3-mediated currents were evoked in each neuron by applying UV flashes of varying intensities (Fig. 12A). The resulting IP_3 concentration-response curve was then constructed and fitted with a logistic equation to determine the EC_{50} and maximal I_{IP3} values. We found that the average EC_{50} value was significantly lower in amphetamine-treated rats compared to controls (Fig. 12B and C), while there was no difference in the maximal I_{IP3} amplitude (Fig. 12D), resulting in a leftward shift in the IP_3-concentration response curve (Fig. 12B). These results demonstrate that repeated amphetamine exposure increases IP_3 sensitivity in VTA dopamine neurons.

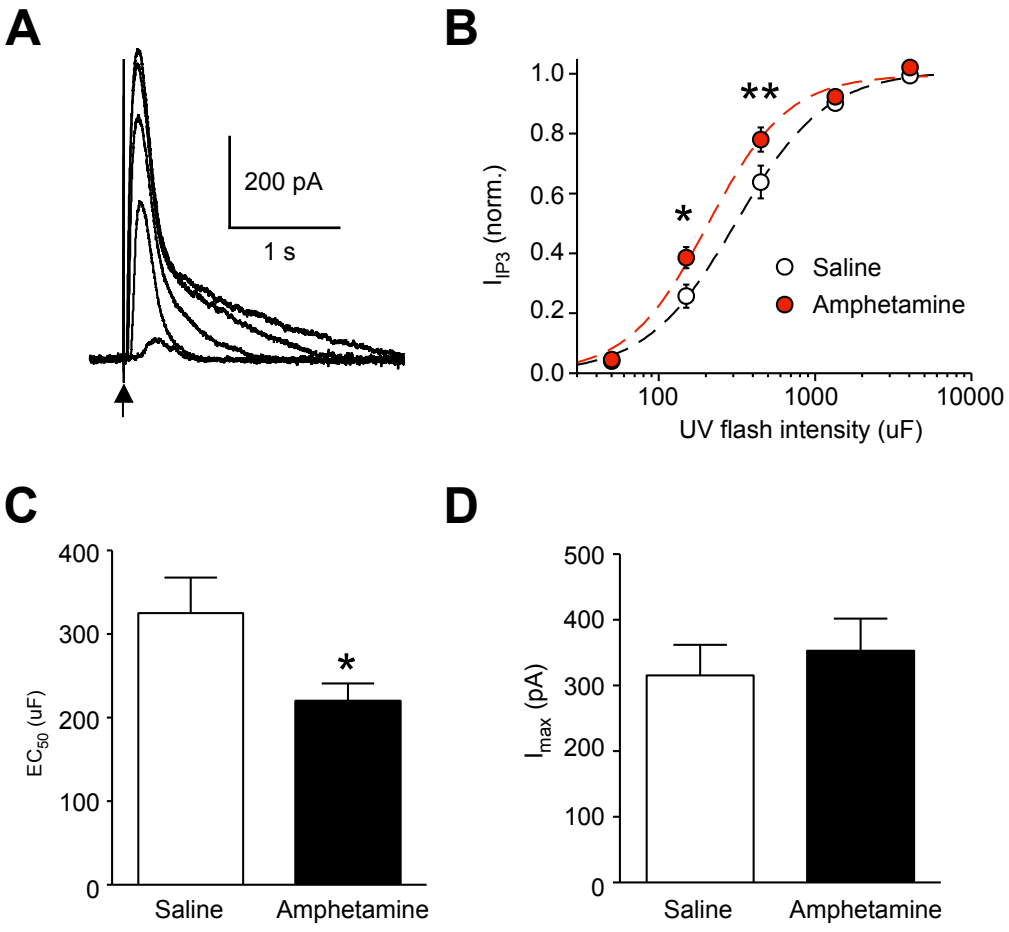

Figure 12. Repeated amphetamine exposure increases IP_3 sensitivity.

(A) Traces of I_{IP3} evoked with different UV pulse intensities (50, 150, 450, 1350, and 4050 µF) in VTA neurons from an amphetamine-treated rat. These cells were loaded with caged IP_3 (100 µM). UV flashes were applied at the time indicated by the arrow. (B) Averaged concentration (UV flash intensity)-response (I_{IP3}) curves from saline and amphetamine-treated mice. The I_{IP3} amplitude was normalized to the maximal value (estimated from fit to a logistic equation) in each cell (saline group: 9 cells from 6 rats, amphetamine group: 10 cells from 8 rats; group: $F_{1,68} = 5.62$, $p < 0.05$; flash intensity: $F_{4,68} = 530$, $p < 0.001$; group · flash intensity: $F_{4,68} = 3.25$, $p < 0.05$, mixed two-way ANOVA). Dashed lines are fit to a logistic equation. $^{**}p < 0.05$, $^{*}p < 0.01$ versus control. (C) Summary bar graph showing that EC_{50} values were reduced in amphetamine-treated mice ($t_{17} = 2.30$, $p < 0.05$, unpaired t test). (D) The maximal I_{IP3} amplitude was not altered by *in vivo* ethanol treatment.

3.2.2 *In vivo* amphetamine exposure enhances IP_3-mediated Ca^{2+} signaling via PKA

As with ethanol and other drugs of abuse, exposure to amphetamine has been shown to cause upregulation of the cAMP-PKA pathway in the VTA and other brain regions (E. J. Nestler and G. K. Aghajanian, 1997). To determine whether increased phosphorylation of IP_3 receptors by PKA underlies the enhanced IP_3 receptor sensitivity and mGluR-mediated facilitation of $I_{K(Ca)}$ seen in the amphetamine treated animals, we tested the effect of a PKA inhibitor H89 (10 μM) on the ability of IP_3 to facilitate single AP-evoked $I_{K(Ca)}$ in treated animals. In these experiments, dopamine neurons were filled with a low concentration of caged IP_3 (25 μM) and subjected to a low-intensity UV flash (100 μF) that produced no measurable outward current by itself. This subthreshold flash caused significant facilitation of $I_{K(Ca)}$ when applied 50 ms prior to an AP (Fig. 13A-C). The flash intensity was held constant in order to compare the magnitude of $I_{K(Ca)}$ facilitation caused by the same concentration of IP_3 in different cells. IP_3-induced facilitation of $I_{K(Ca)}$ was significantly larger in amphetamine-treated rats compared to saline-treated controls (Fig. 13 A-C). Treatment with the PKA inhibitor H89 (10 μM, >1 hr preincubation plus intracellular application through patch pipette) reversed the increase in $I_{K(Ca)}$ facilitation following *in vivo* amphetamine exposure (Fig. 13B-C). IP_3-induced facilitation of $I_{K(Ca)}$ could be restored in the presence of H89 by increasing the intensity of the UV flash (200-500 μF) to release a higher concentration of IP_3 (Fig. 13 D), consistent with the idea that H89 modulates sensitivity of IP_3Rs to IP_3. Taken together, these data

suggest that a PKA-mediated increase in IP_3R sensitivity underlies the enhanced mGluR-mediated facilitation of AP-evoked Ca^{2+} release found in amphetamine animals.

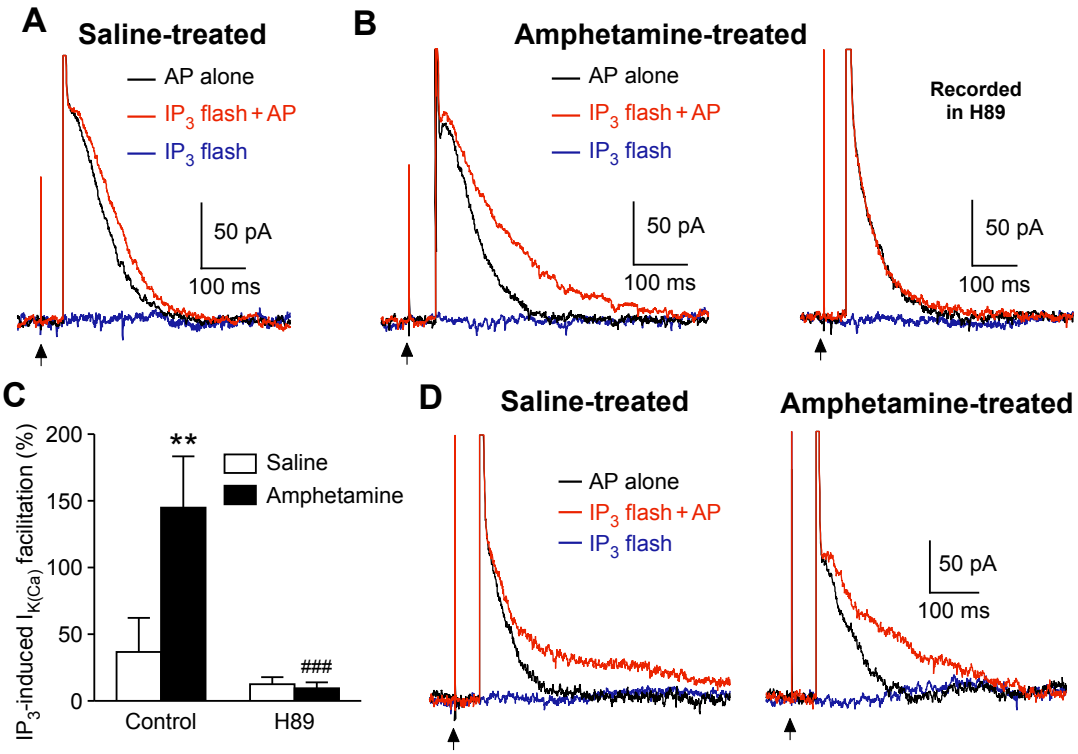

Figure 13. *In vivo* amphetamine exposure enhances IP_3-mediated Ca^{2+} signaling via PKA.

(A) Sample traces of $I_{K(Ca)}$ from saline and amphetamine treated rats, evoked either alone or paired with flash photolysis of IP_3 (25 μM) induced with a low intensity UV pulse (100 μF) applied 50 ms before the depolarization (timing of flash marked by the arrowhead). Response to UV pulse alone is shown in blue. Traces on the right show the blockade of IP_3-induced facilitation of $I_{K(Ca)}$ by H89 (10 μM) in amphetamine-treated animals. (B) Summary bar graph showing IP_3-induced facilitation of $I_{K(Ca)}$ in saline and amphetamine-treated rats under control recording conditions (saline: n = 7 cells from 3 rats, amphetamine: n = 8 cells from 6 rats, **$p < 0.001$ vs. saline group) or in H89 (saline: n = 9 cells from 4 rats, amphetamine: n= 8 cells from 4 rats, ###$p < 0.001$ vs. control condition) [group (saline/amphetamine); $F_{(1,28)} = 5.41$, $p < 0.05$: recording condition (control/H89): $F_{(1.28)} = 12.5$, $p < 0.01$; group x recording condition: $F_{(1,28)} = 6.07$, $p < 0.05$; two-way ANOVA]. (C) Sample traces demonstrating that IP_3-induced facilitation of $I_{K(Ca)}$ can be restored in the presence of H89 with a higher flash intensity (200 μF).

Chapter 4: Discussion

Metaplasticity is a higher-order form of plasticity in which synaptic or neuronal activity patterns induce adaptations that alter the ability of synapses to undergo subsequent induction of synaptic plasticity (W. C. Abraham, 2008). Experience with stressful stimuli, enriched environments or addictive drugs have all been shown to induce metaplasticity that may influence learning (W. C. Abraham, 2008; K. Moussawi et al., 2009). The main finding of the present study is that repeated *in vivo* ethanol exposure induces a metaplasticity of NMDA receptor-mediated transmission in VTA DA neurons. This enhanced susceptibility to induction of LTP results from an increase in IP_3 receptor sensitivity, which, in turn, leads to an increase in IP_3-mediated amplification of AP-associated Ca^{2+} signals (Figure 14). Additionally, in a behavioral assay of reward learning, ethanol-treated animals show increased learning of drug-associated cues.

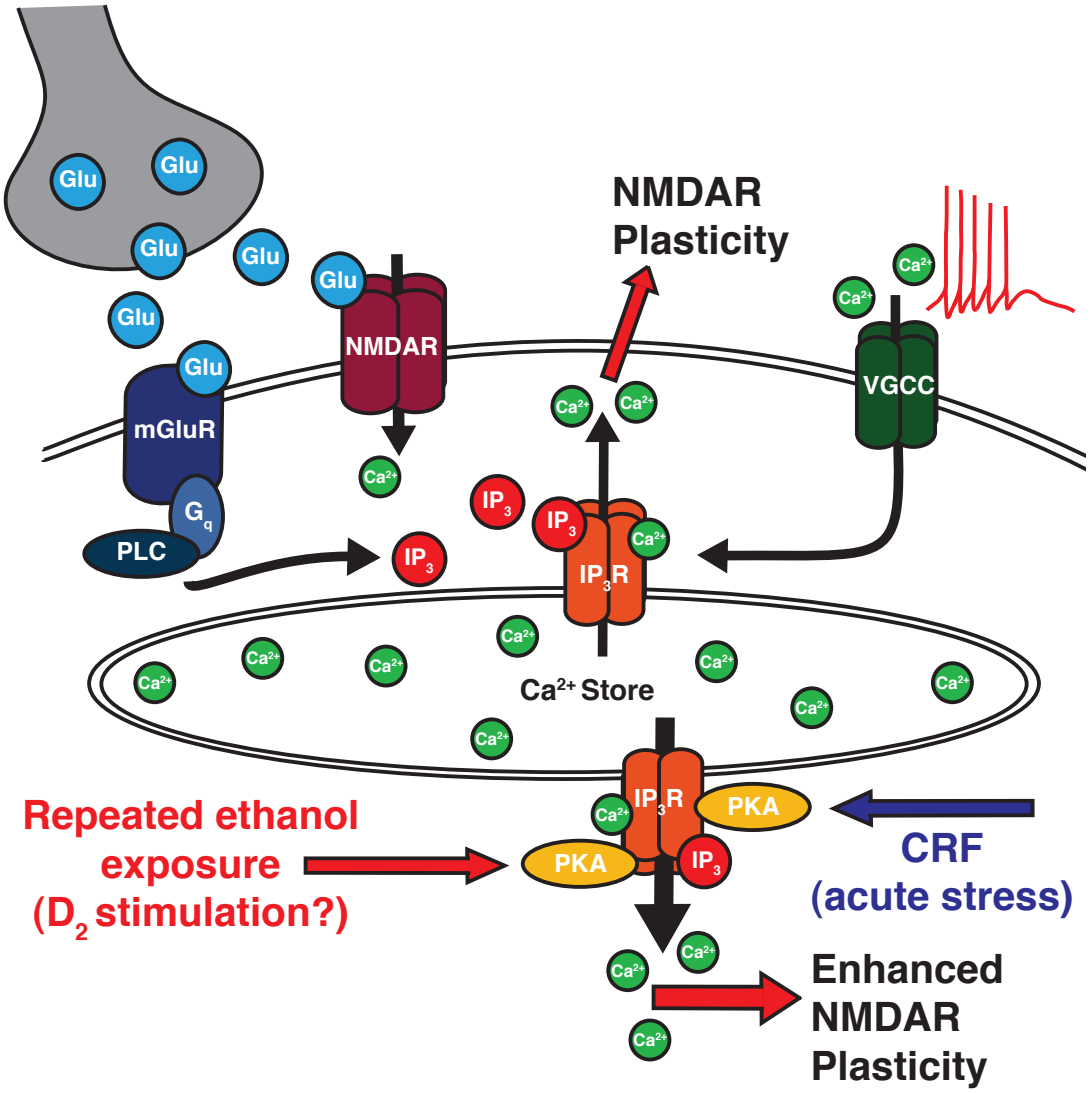

Figure 14. Schematic diagram depicting the interactions between repeated ethanol exposure and CRF on NMDAR LTP.

Both repeated ethanol exposure and acute CRF exposure increase IP_3R-mediated facilitation of AP-associated Ca^{2+} signals in a PKA-dependent manner, thereby enhancing induction of NMDAR LTP.

4.1 REPEATED ETHANOL EXPOSURE ENHANCES IP_3 RECEPTOR SENSITIVITY

Detailed molecular and structural studies have identified two sites at which phosphorylation by PKA enhances IP_3R sensitivity (S. K. Danoff et al., 1991; T. S. Tang et al., 2003; L. E. Wagner, 2nd et al., 2008). The increase in IP_3-induced Ca^{2+} signaling observed in ethanol-treated mice is most likely due to increased PKA phosphorylation of IP_3Rs, as it can be mimicked by activation of the cAMP/PKA pathway by forskolin and reversed by the PKA inhibitor H89. Upregulation of the cAMP/PKA pathway, presumably resulting from chronic activation of G_i-coupled receptors, such as D_2 dopamine receptors or μ-opioid receptors, appears to be a common neuroadaptation observed in the mesolimbic system and other brain areas following exposure to different drugs of abuse (S. E. Hyman et al., 2006). Indeed, ethanol is known to elevate DA levels within the VTA, activating D_2 autoreceptors on DA neurons (Q. S. Yan et al., 1996; R. R. Kohl et al., 1998). Ethanol-induced DA release in the VTA has also been shown to activate presynaptic D_1 receptors on glutamatergic terminals, which may further increase somatodendritic dopamine release via glutamatergic excitation of dopamine neurons (C. Deng et al., 2009; C. Xiao et al., 2009). Interestingly, changes in gene expression and protein levels of multiple components of the cAMP/PKA pathway have been found in tissue samples from human alcoholic brain (M. Yamamoto et al., 2001; R. D. Mayfield et al., 2002). Furthermore, a similar PKA-dependent increase in IP_3R sensitivity in

dopamine neurons was observed in response to *in vivo* exposure to amphetamine, which also increases somatodendritic DA release within the VTA (P. W. Kalivas et al., 1989; L. K. Dobbs and G. P. Mark, 2008), where it presumably activates D_2 autoreceptors as it does in SNc (N. B. Mercuri et al., 1989). Thus, repetitive stimulation of D_2 autoreceptors as a consequence of drug-induced dopamine release within the VTA may be the cellular mechanism mediating the enhancement of IP_3R function seen in this study. Interestingly, a PKA-dependent increase in IP_3R sensitivity may be a common adaptive response to other drugs of abuse as well, as administration of cocaine, nicotine and opioid agonists also increase DA levels in the VTA (M. Yoshida et al., 1993; M. E. Reith et al., 1997).

Importantly, the basal I_{KCa}, measured when evoking APs alone, was not altered by repeated ethanol exposure or acute modulation of the camp/PKA pathway with forskolin, CRF, or H89. The effect of treatment with ethanol, amphetamine, or drugs altering PKA activity on Ca^{2+} signaling only becomes apparent when APs are evoked in combination with the generation of IP_3 pharmacologically, synaptically, or directly by flash photolysis.

These differential effects on Ca^{2+} signaling are interesting for several reasons. First, this finding suggests that PKA activity does not significantly affect voltage-gated Ca^{2+} channels responsible for AP-induced Ca^{2+} influx in DA neurons. This is somewhat surprising considering that PKA is known to modulate multiple voltage-gated Ca^{2+} channel subtypes, including L-type Ca^{2+} channels, which are highly expressed in DA neurons (A. C. Dolphin, 1996; A. Rajadhyaksha et al., 2004; C. S. Chan et al., 2007). However, PKA sensitivity of the pore-forming α_1 subunit of L-type channels appears to be determined by alternative splicing and the presence of certain auxiliary subunits (A.

Rajadhyaksha et al., 2004; Y. Liang and S. J. Tavalin, 2007). Currently, the precise splice variant and subunit composition of $Ca_V1.3$ channels found in DA neurons is unknown.

Second, this finding also suggests that SK channels are not affected by ethanol treatment or PKA activity in DA neurons. As measured here, I_{KCa} is entirely sensitive to the SK channel antagonist apamin (G. Cui et al., 2007), therefore any effect of ethanol treatment on SK channel density or function should appear as a change in basal I_{KCa}. Additionally, the maximal SK-mediated current evoked by flash photolysis of caged IP_3 was not different between groups, consistent with the argument that repeated EtOH treatment does not alter SK channels. These findings contrast somewhat with previous reports that PKA phosphorylation causes internalization of SK2 channels in expression systems, as well as in amygdala and hippocampal neurons (Y. Ren et al., 2006; W. C. Abraham, 2008; E. S. Faber et al., 2008; M. T. Lin et al., 2008). This discrepancy is most likely due to the fact that DA neurons predominantly express SK3 channels (J. Wolfart et al., 2001).

Finally, previous studies have found that CRF can activate the phospholipase C (PLC) pathway via $CRFR_2$ in dopamine neurons (M. A. Ungless et al., 2003). As PLC activation can lead to the generation of IP_3, CRF could be expected to facilitate I_{KCa} through this pathway. However, in this study CRF was found to facilitate I_{KCa} only when AP firing was combined with IP_3 flash photolysis, despite the fact that this effect was dependent on $CRFR_2$. This difference my be due to the fact that the Ungless et al. study used 1 μM CRF, whereas this study used 300 nM CRF, suggesting that $CRFR_2$ is more

strongly coupled to the cAMP/PKA pathway as compared to the PLC/PKC pathway in VTA DA neurons.

4.2 INTERACTIONS BETWEEN THE CRF SYSTEM AND ETHANOL-INDUCED NEUROADAPTATIONS

An extensive body of work has implicated stress and the CRF system in the acquisition, expression, and reinstatement of drug-seeking behaviors (Z. Sarnyai et al., 2001; M. Heilig and G. F. Koob, 2007). For example, systemic administration of CRF antagonists can reverse stress-induced enhancement of the acquisition of cocaine CPP (A. S. Kreibich et al., 2009) and reduce ethanol self-administration in dependent animals (C. K. Funk et al., 2006). Recent evidence also indicates direct interactions between CRF and DA systems within the VTA. Indeed, stress-induced CRF release in the VTA can trigger reinstatement of cocaine-seeking behavior (R. A. Wise and M. Morales, 2010), possibly through direct effects of CRF on DA neurons. *In vitro* studies have shown that CRF can influence intrinsic excitability and glutamatergic excitation of DA neurons, either of which could have a profound effect on DA neuron firing and therefore, encoding of reward-related information (M. A. Ungless et al., 2003; M. J. Wanat et al., 2008). As a result of these findings, CRF has been postulated to enhance the reinforcing properties of drugs and their associated stimuli (D. Saal et al., 2003). Consistent with this idea, acute stress has been shown to enhance the acquisition of ethanol, psychostimulant or opiate self-administration (P. V. Piazza and M. Le Moal, 1998). In addition, acute stress more effectively increases opiate self-administration when the stressor and the self-

administration session are closely paired in time and not when the stressor is applied several hours after the end of the session, suggesting that stress can strengthen learned associations that contribute to drug taking behavior (Y. Shaham, 1993).

Our finding that CRF potentiates IP_3-induced facilitation of $I_{K(Ca)}$ via activation of $CRFR_2$ is in agreement with a previous study demonstrating that CRF increases mGluR-mediated intracellular Ca^{2+} release in DA neurons (A. C. Riegel and J. T. Williams, 2008). These CRF effects on Ca^{2+} signaling occur via the cAMP/PKA pathway, which also mediates the effects of repeated *in vivo* exposure to ethanol and amphetamine, as described above. Despite this shared mechanism of action, the enhancement of IP_3 sensitivity by ethanol exposure does not occlude the facilitation of AP-evoked Ca^{2+} signals by CRF. On the contrary, a robust facilitation of IP_3-mediated Ca^{2+} release can be achieved when acute CRF is combined with prior ethanol exposure. As a result, the effect of ethanol exposure on IP_3R function may enhance the ability of stress to impact DA neuron function. Given the importance of intracellular Ca^{2+} release in synaptic plasticity, PKA regulation of IP_3Rs may, therefore, represent an important site of convergence where the stress system interacts with drug-induced neuroadaptations, thereby promoting drug-induced learning and behaviors (Figure 14). In this regard, it should be noted that increased CRF levels are observed during ethanol withdrawal in multiple brain areas of rodents (Z. Sarnyai et al., 2001; M. Heilig and G. F. Koob, 2007) as well as in the cerebrospinal fluid of alcohol-dependent humans (B. Adinoff et al., 1996). Thus, although CRF tone was not detected in VTA slices in the present study, VTA CRF levels may be elevated *in vivo* during ethanol withdrawal.

Aside from the ability to enhance IP_3-mediated Ca^{2+} release, acute CRF has also been shown to transiently potentiate NMDA receptors in VTA DA neurons (M. A. Ungless et al., 2003), an effect that is enhanced following chronic exposure to drugs (J. Hahn et al., 2009). Interestingly, induction of the burst timing-dependent NMDAR LTP examined in this study is dependent upon NMDAR activation in addition to intracellular Ca^{2+} release (M. T. Harnett et al., 2009). As a result, acute CRF release within the VTA could potentially enhance two independent cellular mechanisms critical for the induction of NMDAR LTP in DA neurons. Acting in concert with the enhancement of IP_3R function following repeated EtOH exposure, the CRF system is uniquely positioned to exert a powerful influence over synaptic plasticity in the VTA during the initial phases of drug experience.

4.3 REPEATED ETHANOL EXPOSURE ENHANCES NMDAR PLASTICITY

Accumulating evidence suggests that plasticity at glutamatergic synapses in the VTA contributes to reward learning and addiction (J. A. Kauer and R. C. Malenka, 2007). Extensive studies have shown that *in vivo* exposure to drugs of abuse, including ethanol, causes global potentiation of AMPAR, but not NMDAR, function in VTA DA neurons (M. S. Brodie, 2002; D. Saal et al., 2003; F. W. Hopf et al., 2007; J. A. Kauer and R. C. Malenka, 2007; G. D. Stuber et al., 2008). This generalized increase in the sensitivity of DA neurons to AMPAR-mediated excitation is thought to result in increased DA release in the NAc and other DA neuron projection targets, thereby enhancing activity-dependent synaptic plasticity in those areas (M. E. Wolf et al., 2004). However, as a global effect,

this type of plasticity lacks synapse specificity at the level of the DA neuron and is therefore unlikely to contribute to the acquisition of the cue-specific conditioned burst response seen in DA neurons *in vivo* (W. Schultz, 1998).

In contrast, the present study demonstrates that ethanol-induced neuroadaptations within the VTA facilitate activity-dependent and input specific plasticity of NMDAR-mediated glutamatergic transmission onto DA neurons. Both *in vitro* and *in vivo* studies have shown that NMDAR-mediated transmission plays a critical role in generating burst firing of DA neurons and phasic DA release in target structures (K. Chergui et al., 1994b; Z. Y. Tong et al., 1996a; P. G. Overton and D. Clark, 1997; H. Morikawa et al., 2003; L. S. Zweifel et al., 2009; J. G. Parker et al., 2010). Theoretically, plasticity of NMDARs represents a potential mechanism underlying the acquisition of the conditioned burst response in DA neurons, or behaviorally, the learning of specific reward-predicting cues. This plasticity would potentiate any synaptic inputs activated by environmental stimuli that repeatedly occur coincident with DA neuron burst firing in response to the presentation of a reward. Subsequently, this potentiation would enhance the ability of these stimuli to evoke DA release and drive behavior on their own, thereby encoding the motivational salience of previously neutral stimuli. In this manner, NMDAR LTP in DA neurons would promote associative learning of specific cues related to drug experience.

Previous studies have shown that the magnitude of facilitation of AP-evoked Ca^{2+} signaling by synaptic stimulation is positively correlated with the magnitude of NMDAR LTP (M. T. Harnett et al., 2009). In this study, neurons from EtOH treated animals displayed significantly greater synaptic facilitation of I_{KCa}, which again correlated well

with the magnitude of LTP. It is therefore likely that increased facilitation of burst-evoked Ca^{2+} signaling during the pairing protocol underlies the increased susceptibility to NMDAR LTP in EtOH treated animals (Figure 14).

NMDARs are thought to play a critical role in triggering phasic DA neuron responses to reward-predicting cues (L. A. Sombers et al., 2009; L. S. Zweifel et al., 2009). Interestingly, *in vivo* exposure to ethanol and other drugs of abuse has been shown to impair LTP induction at GABAergic synapses in VTA dopamine neurons [(Y. Z. Guan and J. H. Ye, 2010; J. L. Niehaus et al., 2010), but also see (M. Melis et al., 2002)]. Since GABAergic inhibition can effectively suppress NMDAR-induced burst firing (C. J. Lobb et al., 2010), these two forms of metaplasticity, i.e., enhancement of NMDAR LTP and impairment of GABA LTP, may work together to promote the development of conditioned DA neuron responses to environmental stimuli associated with drug experience.

4.4 REPEATED ETHANOL EXPOSURE ENHANCES LEARNING OF DRUG ASSOCIATIONS

NMDAR activation in the VTA is required for the learning of drug-associated cues assessed with a CPP paradigm (G. C. Harris et al., 2004; L. S. Zweifel et al., 2008). In accordance with this idea, acute ethanol exposure, which inhibits NMDARs (D. M. Lovinger et al., 1989), has been shown to interfere with CPP when administered immediately before conditioning sessions (C. L. Cunningham and C. M. Gremel, 2006). In contrast, our CPP experiments show that the same repeated ethanol exposure that enhances NMDAR LTP also enhances subsequent learning of cocaine-associated cues

days after the last ethanol treatment. Previous studies performing long-term ethanol pre-exposure did not find an enhancement of cocaine or ethanol CPP (G. Le Pen et al., 1998; G. D. Busse et al., 2005). However, these studies both used significantly different ethanol treatment protocols than the one used here, involving much fewer ethanol injections or voluntary drinking of ethanol-containing solutions in which drinking is spread across 24 hrs and blood alcohol levels are not likely to reach high levels. These discrepancies suggest that fewer doses or more gradual intake of ethanol may not be sufficient to induce the neuroadaptations underlying the enhanced CPP seen in the present study.

This type of "cross-sensitization" of learning shown in our study may account for the concurrent use of different types of drugs frequently seen in alcoholics and drug addicts (E. J. Pennings et al., 2002; J. A. Dani and R. A. Harris, 2005). Indeed, a similar effect to that described here has been demonstrated in other experiments using CPP for environments paired with the same or different drugs (B. T. Lett, 1989; T. S. Shippenberg and C. Heidbreder, 1995), an effect that is NMDAR-dependent, as injection of NMDAR antagonists into the VTA can block the enhancement of morphine CPP by prior treatment with cocaine (J. A. Kim et al., 2004). This effect can also be seen using measures of drug-related learning other than CPP, as prior exposure to amphetamine or nicotine increases the speed of acquisition of operant responding for cocaine (B. A. Horger et al., 1992). These findings are often interpreted as evidence that prior drug exposure leads to sensitization of motivation for rewards (T. E. Robinson and K. C. Berridge, 2003). While it is likely that this so-called "incentive sensitization" contributes to these effects, the

present findings provide support for the idea that an enhancement of the cellular mechanisms underlying associative learning between environmental cues and rewarding stimuli may also contribute, as has previously been suggested (T. E. Robinson and K. C. Berridge, 1993; C. Nocjar and J. Panksepp, 2002; T. E. Robinson and K. C. Berridge, 2003). Sensitization of IP_3Rs in the VTA may thus be a common drug-induced neuroadaptation among alcohol and other drugs of abuse that acts to promote the formation of powerful memories of stimuli encountered during the initial days to weeks of drug experience and withdrawal.

4.5 FUTURE DIRECTIONS

A major conclusion of this dissertation is that an ethanol-induced enhancement of susceptibility to NMDA receptor LTP promotes the future formation of drug-cue associations. This hypothesis relies on the assumption that the LTP of NMDA receptor mediated transmission onto DA neurons studied here does in fact underlie reward learning *in vivo*. Although past work has demonstrated that blocking NMDA receptors or PKA activity in the VTA inhibits CPP (G. C. Harris et al., 2004; K. C. Ahn et al., 2010), it will be critical for subsequent experiments to examine the role of this DA neuron LTP in reward learning more directly. Ideally, future studies would demonstrate that intracranial injections of mGluR antagonists into the VTA interfere with reward learning when given during conditioning.

The findings reported here describing the interaction between CRF and repeated ethanol on the Ca^{2+} signals underlying NMDAR LTP also raise numerous questions that

could be addressed through future studies. The large increase in IP_3-mediated facilitation of AP-associated Ca^{2+} signals that results when acute CRF is combined with previous ethanol exposure suggest that CRF would have profound effects on induction of NMDAR LTP in DA neurons. Perfusion of CRF during LTP induction should produce an even greater enhancement of LTP in ethanol-treated animals. Furthermore, as CRF perfusion should be analogous to acute stress, it would be interesting to determine how acute stress and repeated ethanol exposure interact to affect reward learning in behavioral experiments.

Additionally, future work will be necessary to test the hypothesis that chronic D_2 receptor activation underlies the ethanol or amphetamine induced increase in IP_3R sensitivity. This could be done by administering a D_2 receptor antagonist directly into the VTA in conjunction with each ethanol or amphetamine treatment. If this hypothesis is correct, such a treatment should block the effects of ethanol or amphetamine presented here. In this regard, it would also be important to determine whether other drugs that increase DA levels in the VTA, such as nicotine or opiates (M. Yoshida et al., 1993; M. E. Reith et al., 1997), would also produce this metaplasticity of NMDA receptor LTP. If drug-induced enhancements of IP_3R sensitivity prove to be a universal adaptation in response to exposure to multiple addictive drugs, the mechanisms involved in this adaptation could represent potentially powerful and novel therapeutic targets for the treatment of addiction.

4.6 CONCLUSIONS

DA neuron burst firing and the resulting phasic DA release is thought to act as a reinforcement signal for reward learning by modulating synaptic plasticity in downstream target structures. Rather than acting as a simple reward signal however, DA neuron burst firing encodes reward prediction error, thereby promoting the learning of reward contingencies. Over the course of behavioral training DA neuron burst responses become conditioned, transitioning from the reward itself to cues that reliably predict the availability of the reward. In this way, DA neuron burst firing can attribute motivational salience to previously neutral stimuli and learned associations between these cues and the reward are formed. The persistent nature of addiction is thought to derive, in part, from the powerful influence of long-term memories of associations between drugs and their related cues learned during drug experience.

The primary findings of this dissertation demonstrate that ethanol experience produces a metaplasticity of NMDA receptor-mediated transmission onto VTA DA neurons. Neuroadaptations induced by repeated ethanol exposure enhance the mechanisms involved in intracellular Ca^{2+} release such that DA neurons exhibit greater NMDA receptor LTP (Fig. 14). Furthermore, animals in this study exhibit increased learning of cocaine-associated cues following repeated ethanol exposure. These findings suggest that an enhanced susceptibility to NMDA receptor LTP may promote the formation of the strong drug-cue associations that characterize addiction and drive drug-taking behavior.

It is well established that ethanol inhibits NMDAR receptor function and suppresses many forms of synaptic plasticity that are dependent on NMDA activation in

the CNS (D. M. Lovinger et al., 1989; D. Ron, 2004). While long-term ethanol exposure has been shown to produce an upregulation of NMDAR function/expression in other brain regions, it is not clear whether this leads to an enhancement of NMDAR-dependent synaptic plasticity (D. N. Stephens et al., 2005; J. X. Xia et al., 2006; J. Sabeti and D. L. Gruol, 2008). The findings in this dissertation are the first to demonstrate an enhancement of LTP in VTA DA neurons following ethanol exposure. As this LTP is both activity-dependent and synapse specific, it represents a candidate cellular mechanism for associative learning of cue-reward relationships. Thus, this novel effect of ethanol on NMDA receptor LTP provides an important insight into the plasticity involved in the learning component of addiction.

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